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(57) Abstract

The present invention provides methods for treating autoimmune diseases in mammals and for preventing or treating transplantation rejection in a transplant recipient. The methods of treatment involve the use of drugs capable of suppressing expression of MHC Class I molecules. In particular the use of the drug methimazole to suppress expression of MHC Class I molecules in the treatment of autoimmune diseases and the prevention or treatment of rejection in a transplant recipient is disclosed. In addition *in vivo* and *in vitro* assays are provided for the assessment and development of drugs capable of suppressing MHC Class I molecules. In addition, this invention relates to nucleic acid and amino acid sequences for proteins capable of modulating MHC Class I expression.

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Title of the Invention

METHODS OF ASSESSING MHC CLASS I EXPRESSION AND PROTEINS CAPABLE OF MODULATING CLASS I EXPRESSION.

This is a continuation-in-part application of U.S.S.N. 08/480,525 filed June 7, 1995 which is a continuation of U.S.S.N. 08/073,830 filed June 7, 1993, which is herein incorporated by reference in its entirety.

Field of the Invention

This invention is in the field of treatment of autoimmune diseases and transplantation rejection in a mammal. More specifically, this invention relates to methods for treating and preventing these diseases using drugs capable of suppressing expression of the major histocompatibility complex (MHC) Class I molecules and to methods for the development or assessment of drugs that are capable of suppressing MHC Class I expression. This invention also relates to genes and their corresponding proteins capable of modulating MHC Class I expression.

Background of the Invention

A primary function of the immune response is to discriminate self from non-self antigens and to eliminate the latter. The immune response involves complex cell to cell interactions and depends primarily on three major cell types: thymus derived (T) lymphocytes, bone marrow derived (B) lymphocytes, and macrophages. The immune response is mediated by molecules encoded by the major histocompatibility complex (MHC). The two principal classes of MHC molecules, Class I and Class II, each comprise a set of cell surface glycoproteins ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr, A.I. (eds), Appelton and Lange, Norwalk, Connecticut/San Mateo, California). MHC Class I molecules are found on virtually all somatic cell types, although at different levels in different cell types. In contrast, MHC Class II molecules are normally expressed only on a few cell types, such as lymphocytes, macrophages and dendritic cells.

Antigens are presented to the immune system in the context of Class I or Class II cell surface molecules; $CD4^+$ helper T-lymphocytes recognize antigens in association with Class II MHC molecules, and $CD8^+$ cytotoxic lymphocytes (CTL) recognize antigens in association with Class I gene products. It is currently believed that MHC Class I molecules function primarily as the targets of the cellular immune response, whereas the Class II molecules regulate both the humoral and cellular immune response (Klein, J. and Gutze, E., (1977) "Major Histocompatibility Complex" Springer Verlag, New York; Roitt, I.M. (1984) Triangle, (Engl Ed) 23:67-76; Unanue, E.R. (1984) Ann. Rev. Immunology, 2:295-428). MHC Class I and Class II molecules have been the focus of much study with respect to research in autoimmune diseases because of their roles as mediators or initiators of the immune response. MHC-Class II antigens have been the primary focus of research in the etiology of autoimmune diseases, whereas MHC-Class I has historically been the focus of research in transplantation rejection.

Graves' disease is a relatively common autoimmune disorder of the thyroid. In Graves' disease, autoantibodies against thyroid antigens, particularly the thyrotropin receptor, alter thyroid function and result in hyperthyroidism ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr, A.I. (eds), Appelton and Lange, Norwalk, Connecticut/San Mateo, California: pages 469-470). Thyrocytes from patients with Graves' disease have aberrant MHC-Class II expression and elevated MHC Class I expression. (Kohn et al., (1992) In "International Reviews of Immunology," Vol. 912:135-165).

Thionamide therapy has historically been used to treat Graves' disease. The most commonly used thionamides are methimazole (MMI), carbimazole (CBZ) and propyl-thiouracil (PTU). These thionamides contain a thiourea group; the most potent are thioureylenes (W.L. Green

(1991) In Werner and Ingbar's "The Thyroid": A Fundamental Clinical Text" 6th edition, L. Braverman and R. Utiger (eds), J.B. Lippincott Co. page 324). The thionamides restore a euthyroid state by inhibiting thyroid peroxidase catalyzed formation of the thyroid hormones produced by the thyroid stimulating autoantibody stimulated thyroid (Solomon, D.H. (1986) In "Treatment of Graves' Hyperthyroidism". Ingbar, S.H., Braverman, L.E. (eds) The Thyroid: JB Lippincott Co., Philadelphia, Pennsylvania, p. 987-1014; Cooper, D.S. (1984) N. Engl. J. Med., 311: 1353-1362; Cooper, D.S. (1991) Treatment Of Thyrotoxicosis. In Werner And Ingbar's The Thyroid: A Fundamental and Clinical Text," 6th edition. L. Braverman and R. Utiger (eds), J. B. Lippincott Co. pages 887-916). It has been reported that MMI and PTU can inhibit peroxidase-dependent enzymes in the kidney and that MMI can inhibit gastric peroxidase in rat gastric mucosa (Zelman, S.J. et al., (1984) J. Lab. Clin. Med. 104:185-192; Bandyopadhyay, U. et al., (1992) Biochem J. 284:305-312). PTU has also been reported to inhibit hepatoxicity associated with alcoholism (Orrego, H. et al., (1987) N. Engl. J. of Med. 317:1421-1427). Thionamides have been used to treat Graves' patients for extended periods of time with the majority of patients experiencing no complication related to this therapy (Cooper, D.S. (1991) Treatment Of Thyrotoxicosis. In Werner And Ingbar's The Thyroid: A Fundamental and Clinical Text," 6th edition. L. Braverman and R. Utiger (eds), J. B. Lippincott Co. pages 887-916). Allergic reactions, including such symptoms as fever, rash, urticaria, occur in 1-5% of patients. Toxic reactions to thionmide treatments are rare, occurring in only 0.2 to 0.5% of the patients (Cooper, D.S. (1991) Treatment Of Thyrotoxicosis. In Werner And Ingbar's The Thyroid: A Fundamental and Clinical Text," 6th edition. L. Braverman and R. Utiger (eds), J. B. Lippincott Co. pages 887-916).

In addition to the effect of thionamides on thyroid hormone synthesis, it was recognized that thionamide therapy in Graves' disease was associated with a reduction in thyroid autoantibodies (Cooper, D.S. (1982) N. Clin. Endocrinol. Metab. 29:231-238; Kuzuya, N. et al., J. Clin. Endocrinol. Metab. 48:706-714; Bech, K. and Madsen, S.N. (1980) Clin Endocrinol (Oxf) 13:417-26; Hallengren, B. et al. (1980) J. Clin. Endocrinol. Metab 51:298-301; Cooper, D.S. (1991) Treatment Of Thyrotoxicosis. In Werner And Ingbar's "The Thyroid: A Fundamental and Clinical Text," 6th edition. L. Braverman and R. Utiger (eds), J. B. Lippincott Co. pages 887-916). Studies on the mechanism by which thionamides exert this effect are contradictory. One hypothesis suggests that the thionamides act directly on thyroid follicular cells and that the subsequent modulation in thyroid activity results in the immune effects (Volpe et al., (1986) Clin. Endocrinol. 25:453-462). A second hypothesis suggests that thionamides act directly on lymphocytes, particularly thyroid lymphocytes (Weetman, A.P. (1992) Clin Endocrinol. 37:317-318; McGregor, A.M. (1980) Brit. Med. J., 281:968-969). It has also been suggested that MMI interferes with antigen handling by accessory cells because these cells possess a peroxidase enzyme system (Weetman, A.P. (1983) Clin. Immuno. and Immunopath, 28:39-45). The current consensus appears to be that the therapeutic action of the thionamides, including the immunosuppressive effects, is thyroid specific and intra-thyroidal (D.S. Cooper (1991) Treatment Of Thyrotoxicosis. In Werner And Ingbar's The Thyroid: A Fundamental and Clinical Text," 6th edition. L. Braverman and R. Utiger (eds), J. B. Lippincott Co. pages 888-889).

Results of studies involving the use of MMI in the treatment of diabetes are also contradictory. Hibbe, T. et al., (1991); Diabetes Res. and Clin. Practice 11:53-58, report that MMI enhances the development of

streptozotocin-mediated diabetes in mice. In contrast, Waldhausl, W. et al. (1987) Akt. Endokrin. Stoffw. 8, 119 (abstract) report enhanced remission in 54% of 11 patients treated with MMI shortly after diagnosis of type I diabetes, basing their therapy on reputed effects of MMI on T helper cells. These authors reported no change in the levels of Class I and Class II antigens and it is unclear whether the effect was due to MMI or natural remission of the disease over the 9 month "honeymoon" period. In the BB rat, MMI depressed spontaneous development of thyroiditis but did not reduce the incidence of diabetes (Allen, F.M. et al., (1986) Am. J. Med. Sci., 292: 267-271; Braverman, L.E. et al., (1987) Acta. Endocrinol. (Coppenhagen) Suppl. 281: 70-76).

Saji, M. et al. (1992a); Proc. Natl. Acad. Sci. U.S.A. 89:1944-1948 describe hormonal regulation of MHC-class I genes in the rat thyroid cell line, FRTL-5. Treatment of the FRTL-5 cell line with thyroid stimulating hormone (TSH) resulted in decreased transcription of MHC class I DNA and reduced cell surface levels of MHC Class I antigens. Recently, a report by Saji, M. et al., (1992b) J. Clin. Endocrinol. Metab. 75:871-878, demonstrated that agents such as serum, insulin, insulin-like growth factor - I (IGF-1), hydrocortisone and thyroid stimulating thyrotropin receptor autoantibodies from Graves' patients decrease MHC-Class I gene expression in that FRTL-5 cells. In addition, treatment of the FRTL-5 cells with MMI or high doses of iodide resulted in decreased MHC Class I gene expression. The effect of MMI on reduction of MHC Class I expression was shown to be at the level of transcription and was additive with thyroid stimulating hormone and other hormones which normally suppress Class I in these cells. Saji, M. et al. (1992b) J. Clin. Endocrinol. Metab. 75:871-878, suggest a new mechanism by which MMI may act in the thyroid during treatment of Graves' disease; no extrapolation was made to any other

autoimmune diseases. Prior to these studies it was known that Rous sarcoma virus, adenoviruses 12 and 2 and certain Gross viruses reduced expression of MHC Class I; however, SV40, Rad LV, and Mo MuLV viruses can increase Class I MHC expression (Singer & Maguire (1990) Crit. Rev. in Immun. 10:235-257).

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that, like Graves', has a relatively high rate of occurrence. SLE affects predominantly women, the incidence being 1 in 700 among women between the ages of twenty and sixty ("Cellular and Molecular Immunology (1991) Abbus, A.K., Lichtman, A.H., Pober, J.S. (eds); W.B. Saunders Company, Philadelphia: page 360-370). SLE is characterized by formation of a variety of autoantibodies and by multiple organ system involvement ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr, A.I. (eds), Appelton and Lange, Norwalk, Connecticut, San Mateo, California: pages 438-443). Current therapies for treating SLE involve the use of corticosteroids and cytotoxic drugs, such as cyclosporin. Immunosuppressive drugs such as cyclosporin, FK506, or rapamycin suppress the immune system by reducing T cell numbers and function (Morris, P.J. (1991) Curr. Opin. in Immun., 3:748-751). While these immunosuppressive therapies alleviate the symptoms of SLE, and other autoimmune diseases, they have numerous severe side effects. In fact, extended therapy with these agents may cause greater morbidity than the underlying disease.

Women suffering from SLE who have breast cancer face particular difficulties. These individuals are immunosuppressed as a result of corticosteroid and cytotoxic drug treatment for SLE; radiotherapy for the treatment of the cancer would additionally enhance the immunosuppressed state. Further, radiation therapy, a current method of choice can exacerbate disease expression or induce severe radiation complications. For these

individuals, alternative therapies that would allow for simultaneous treatment of SLE and the cancer are greatly needed. In general, alternative therapies or new methods of assessing the therapeutic potential of drugs for treating autoimmune diseases are greatly needed.

As is true for autoimmune diseases, there is a great need for different ways of treating or preventing transplantation rejection. Transplantation rejection occurs as a result of histoincompatibility between the host and donor; it is the major obstacle in successful transplantation of tissues. Current treatment for transplantation rejection, as for autoimmune disease, involves the use of a variety of immunosuppressive drugs and corticosteroid treatment.

Faustman et al., (PCT International Application No. 92/04033) identify a method for inhibiting rejection of a transplanted tissue in a recipient animal by modifying, eliminating, or masking the antigens present on the surface of the transplanted tissue. Specifically, this application suggests modifying, masking, or eliminating human leukocyte antigen (HLA) Class I antigens. The preferred masking or modifying drugs are F(ab)' fragments of antibodies directed against HLA-Class I antigens. However, the effectiveness of such a therapy will be limited by the hosts' immune response to the antibody serving as the masking or modifying agent. In addition, in organ transplantation this treatment would not affect all of the cells because of the perfusion limitations of the masking antibodies. Faustman et al. disclose that fragments or whole viruses be transfected into donor cells, prior to transplantation into the host, to suppress HLA Class I expression. Use of whole or fragments of virus presents potential complications to the recipient of such transplanted tissue since some viruses, SV40 in particular, can increase Class I expression

(Singer and Maguire (1990) Crit. Rev In Immunol. 10:235-237, TABLE 2).

Durant et al. (British Patent No. 592, 453) identify isothiourea compositions that may be useful in the treatment of autoimmune diseases and host versus graft (HVG) disease and assays for assessing the immunosuppressive capabilities of these compounds. However, this study does not describe MMI or the suppression of MHC Class I molecules in the treatment of autoimmune diseases.

U.S. Patents 5,010,092 and 5,097,441 describe a method for reducing nephrotoxicity resulting from administration of an antibiotic in a mammal by coadministration of the antibiotic with either MMI or CBZ. These patents neither suggest nor teach the use of MMI to suppress MHC Class I expression in the treatment of autoimmune diseases or in the treatment and prevention of transplantation rejection, a then suppression of Class I molecules as a therapeutic indication of drug to be used in treating autoimmune diseases and transplantation rejection.

SUMMARY OF THE INVENTION

This invention relates to methods for treating autoimmune diseases in mammals and for preventing or treating transplantation rejection in a transplant recipient. These methods involve administering to a mammal in need of treatment a drug capable of suppressing expression of MHC Class I molecules. This invention also relates to pretreating transplantable cells, tissues or organs prior to transplantation into a recipient with a drug capable of suppressing MHC Class I molecules. In particular this invention relates to the use of MMI in treating autoimmune diseases in mammals and for preventing or treating transplantation rejection in a transplant recipient. In addition, this invention further includes methods for in vivo and in vitro assays for the

development and assessment of drugs capable of suppressing expression of MHC Class I molecules.

One in vivo method may be comprised of three steps. First, MHC Class I deficient mice are used to evaluate the importance of MHC Class I in a particular experimental autoimmune disease. Second, an animal which is useful as a model for a particular autoimmune disease, either experimentally induced or spontaneous, is exposed to the drug being evaluated. Third, the therapeutic potential of the drug is evaluated by the alleviation of symptoms or signs of the autoimmune disease in the treated animal.

Another in vivo method may be also comprised of three steps. First, a mammalian cell line, tissue or organ is treated with the drug. Second, the treated cells, tissues, or organs are transplanted into a mammal which may also be treated with the drug. Third, the cells are removed from the recipient mammal and cell survival is evaluated.

There are a vareity of methods provided herein for the in vitro assays. In one method the ability of the drug to suppress expression of MHC Class I molecules is assessed by treating mammalian cells with a candidate drug, combining cell extracts from the treated cells with MHC Class I regulatory nucleic acid sequences, detecting formation of a complex between the nucleic acid sequences and proteins from the extract, and comparing alterations in complex formation in extracts from treated and untreated cells. In another in vitro method, the therapeutic potential of the drug may be evaluated by its ability to down regulate Class I transcription in cells. By way of example down regulation of MHC Class I transcripts may be assessed by reporter gene assays.

In yet another in vitro assay the ability of a drug to suppress expression of MHC Class I molecules is

evaluated by its ability to alter expression of proteins capable of modulating expression MHC Class molecules.

Yet another object of the invention is to provide nucleic acid sequences which encode proteins capable of modulating MHC Class I expression.

It is yet another object of this invention to provide a recombinant molecule comprising a vector and all or part of the nucleic acid sequences provided herein which are capable of modulating Class I expression.

It is yet another object of this invention to produce recombinant proteins encoded by all or part of the nucleic acid sequences encoding the proteins capable of modulating MHC Class I.

It is a further object of this invention to provide monoclonal or polyclonal antibodies reactive with those proteins, peptides or portions thereof.

An object of the invention is to provide a method for treating mammals suffering from autoimmune diseases.

Another object of the invention is to provide a method of preventing or treating rejection of a tissue in a transplant recipient.

A further object of the invention is to provide a method for preventing rejection of cells containing a recombinant gene transplanted into a mammal in need of gene therapy.

Another object of the invention is to provide in vivo and in vitro assays for the assessment and development of drugs capable of suppressing MHC Class I molecules.

A further object of the invention is to provide in vivo and in vitro assays that are predictive of the therapeutic usefulness of candidate drugs.

DESCRIPTION OF FIGURES

Figures 1A-1D shows that Class I-deficient mice generate anti-16/6Id antibodies, but not anti-DNA or anti-

nuclear antigen antibodies. Serial two-fold dilutions of sera were assayed by ELISA 10 weeks after immunization. Results are the average of measurements of 5 individual animals and are expressed as OD at 405 nm X 10^3, as a function of serial serum dilutions. Standard deviation values did not exceed 10% of the mean. Sera of 16/6Id-immunized control 129 mice (○), 16/6Id-immunized Class I-deficient mice (◆), and ovalbumin-immunized Class I-deficient mice (●).

1A. Titration of 16/6Id binding in the sera of immunized mice; purified 16/6Id immobilized on plates.

1B. Titration of single-stranded DNA binding in the sera of immunized mice; single-stranded DNA immobilized on plates.

1C. Titration of nuclear antigen binding in the sera of immunized mice; nuclear extract immobilized on plates.

1D. Titration of ovalbumin binding in the sera of immunized Class I-deficient mice (●); ovalbumin immobilized on plates. Sera of Class I-deficient mice which were not immunized with ovalbumin (⊙) are the control in this experiment.

Figures 2A-2D show that Class I-deficient mice do not respond to immunization with monoclonal anti-16/6Id antibody. Serial two-fold dilutions of sera were analyzed by ELISA, 7 weeks after immunization. Results are the average of measurements of 6 animals. Standard deviation value did not exceed 10% of the mean. Sera of anti-16/6Id-immunized control 129 (■) and anti-16/6Id-immunized class I-deficient mice (●)

2A. Titration of anti-16/6Id binding in the sera of immunized mice; purified rabbit polyclonal anti-16/6Id immunoglobulin immobilized on plates.

2B. Titration of single-stranded DNA binding in the sera of immunized mice; single-stranded DNA immobilized on plates.

2C. Titration of 16/6Id binding in the sera of immunized mice; 16/6Id immobilized on plate.

2D. Titration of nuclear antigens binding in the sera of immunized mice; nuclear extract immobilized on plate.

Figures 3A-3B shows immunohistological examination of kidney sections of Class I-deficient (3B) and control 129 (3A) mice injected with 16/6Id. Frozen kidney sections (5 μm thick) were fixed and stained with FITC-conjugated, gamma chain-specific goat anti-mouse IgG (magnification X200). The kidney sections shown are from one individual in each group and are representative of that group.

Figures 4A-4D. Figures 4A and 4B show the appearance of anti-16/6Id and anti-DNA antibodies in mice exposed to a single immunization and boost with a human monoclonal anti-DNA antibody bearing the 16/6Id. Figures 4C and 4D show titers of the anti-16/6Id and anti-DNA antibodies in mice 21 1/2 weeks after treatment with MMI.

4A. Shows the titer of anti-16/6Id antibodies in mice prior to treatment with MMI. Control Balb/c mice which received no immunization (⊡); 16/6Id immunized mice which will or will not be treated with MMI or MMI plus thyroid hormone, specifically thyroxine (T_4) (◇, ◆, □).

4B. Shows the titer of anti-DNA antibodies in mice prior to treatment with MMI. Designations are the same as in (A).

4C. Shows the titer of anti 16/6Id antibodies in mice after treatment with MMI or MMI and thyroxine (T_4). Control animals immunized with 16/6Id but receiving no treatment (⊡); animals immunized with 16/6Id then treated with 60 days of MMI (◆), or with 60 days of MMI and T_4 (◇); animals which were not immunized with 16/6Id but were treated with 60 days of MMI (■) or animals treated with 60 days of MMI and T_4 (□).

4D. Shows the titer of anti DNA antibodies in mice after treatment with MMI or MMI and thyroxine (T_4). Designations are the same as in (C).

Figure 5 shows the relative white blood cell (WBC) count as a percentage of the WBC in 16/6Id-treated control animals with no exposure to MMI or thyroxine (T_4) (■); in 16/6Id-treated animals exposed to MMI(O); and 16/6Id-treated animals exposed to MMI and T_4(□) at 3 months, 5 months and 8 months after the boost.

Figures 6A-6B show the development of immune complexes in the kidneys of 16/6Id-treated mice treated with MMI (6B) versus 16/6Id-treated animals not treated with MMI (6A).

Figures 7 A-D shows the effect of MMI treatment on lymphocyte populations during experimental SLE disease. The experimental SLE disease resulted from treatment with 16/6Id.

7A. Shows the distribution of the lymphocyte populations in mice immunized with 16/6Id (▨); mice immunized with 16/6Id and treated with MMI and thyroxine (T_4) (▩); mice immunized with 16/6Id and treated with T_4 (▨); mice immunized with 16/6Id and treated with MMI (▦); and mice immunized with 16/6Id and administered placebo (▨).

7B. Shows the levels of Class I on T cells over time in mice immunized with 16/6Id (▨) and mice immunized with 16/6Id and treated with MMI (▩).

7C. Shows the levels of Class I on B cells over time in mice immunized with 16/6Id (▨) and mice immunized with 16/6Id and treated with MMI (▩).

7D. Shows the levels of Class II on B cells over time in mice immunized with 16/6Id

(▽) and mice immunized with 16/6Id and treated with MMI (■).

Figures 8 shows the effect of 2 months of MMI treatment (15 mg released over 30 days by pellet implantation) on anti-DNA antibody titers in NZBxNZWF1 mice. Control animals (BxWF1) (□) and BxWF1 animals treated with MMI (o). NZBxNZWF1 mice are a mouse model of SLE that develop spontaneous SLE.

Figure 9A-9B shows the sequence of PD1 promoter (SEQ ID NO:1) with the 151 (bold) (bases 54 to 220 of SEQ ID NO:1), 114 (bold and underlined) (bases 221 to 320 of SEQ ID NO:1), 140 (bold and boxed) (bases 321 to 455 of SEQ ID NO:1) and 238 (bold and wavy box) (bases 456 to 692 of SEQ ID NO:1) regions or fragments of the 5' portion of the PD1 promoter. The 238 region (bases 456 to 692 of SEQ ID NO:1) includes an AT rich 105 region (underlined) (bases 588 to 692 of SEQ ID NO:1). The ATG start site is noted by the amino acid 3 letter code starting with Met. The numbers at the right indicate the number of base pairs counting from the first nucleotide in the uppermost line.

Figure 10 shows the silencer and enhancer regions of the 140 fragment (SEQ ID NO:2) with oligonucleotides used to map the region for activity in gel mobility shift assays. The silencer region which is relevant to the MMI effects on complex formation in mobility gel shift assay is noted by the opposites arrows separated by a thyroid transcription factor-2 (TTF-2)-like binding element which is insulin-sensitive. Mapping of silencer-and enhancer-binding sites was by inhibition of complex formation by various double-stranded (ds)-oligonucleotides. A series of ds-oligonucleotides spanning the 140-bp *AvaII-DdeI* DNA fragment was tested for the ability to compete against enhancer and silencer complexes. Of these, the only ones that competed were those contained within the 96-bp segment shown. To determine important residues for binding, variant ds-

oligonucleotides were synthesized and tested for their abilities to inhibit silencer and enhancer complex formation. Boxed regions represent sequences determined by the inhibition studies using the ds-oligonucleotides to be critical for complex formation, dots denote residues identical to the native sequence. For simplicity, only one strand of the ds-oligonucleotide sequence used in competition studies is shown.

Figure 10 (top) shows oligonucleotides used to map the silencer-binding site. Arrows delineate boundaries to the silencer element. Figure 10 (bottom) shows oligonucleotides used to map the enhancer-binding site. Arrows delineate the interrupted, inverted repeat of the enhancer.

Figure 11 shows the alignment of the 114 fragment (SEQ. ID NO:36), 140 fragment (SEQ. ID NO:37) and 105 fragment (SEQ ID NO:35) of the 238 fragment (bases 456 to 692 of SEQ ID NO:1) to show sequence homology. The silencer region is outlined in 140 (SEQ ID NO:37) by the arrows as in Figure 10. All respond to MMI, as does 151. Also identified, as in Figure 10, is the TTF-2 like sequence. The (*) denotes identity with the 140 fragment; the (●) homology with the 140 fragment in at least one other fragment; the (--) denote gaps inserted by the computer to derive the best fit. On the right, the numbers denote the residue in each fragment which is defined in Figure 9 when each is sequentially numbered starting with number one.

Figures 12 A-D show mobility-shift assays using the radiolabelled 140 (bases 321 to 455 of SEQ ID NO:1), 114 (bases 221 to 320 of SEQ ID NO:1) and 151 (bases 54 to 220 of SEQ ID NO:1) fragments noted in Figure 9 and cell extracts from FRTL-5 rat thyroid cells. Cell extracts from treated or untreated FRTL-5 cells were incubated with the radiolabelled DNA fragments, and resulting DNA fragment-protein complexes were electrophoresed in a

polyacrylamide gel and visualized by autoradiography. The complex affected by MMI is denoted A.

12A. Shows the gel mobility shift assays of the 140 radiolabelled fragment (bases 321 to 455 of SEQ ID NO:1): lane 1 contains the 140 radio-labelled fragment (bases 321 to 455 of SEQ ID NO:1) alone; lane 2 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 6H medium and treated with MMI; lane 3 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 6H medium and not treated with MMI; lane 4 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of the 5H medium; lane 5 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and treated with MMI; lane 6 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and treated with thyroid stimulating hormone (TSH); and lane 7 contains cell extracts from FRTL-5 rat thyroid cells maintained in 5H medium and treated with MMI and TSH.

12B. Shows the gel mobility shift assays of the 114 radiolabelled fragment (bases 221 to 320 of SEQ ID NO:1) with FRTL-5 rat thyroid cell extracts. Lane 1 contains the 114 radiolabelled fragment (bases 221 to 320 of SEQ ID NO:1) alone; lane 2 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 6H medium and treated with MMI; lane 3 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 6H medium and not treated with MMI; lane 4 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of the 5H medium; lane 5 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and treated with MMI; lane 6 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and treated with thyroid stimulating hormone (TSH); and lane 7 contains

cell extracts from FRTL-5 rat thyroid cells maintained in 5H medium and treated with MMI and TSH.

12C. Shows the gel mobility shift assays of the 151 radiolabelled fragment with FRTL-5 cell extracts. Lane 1 contains the 151 radiolabelled fragment alone; lane 2 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 6H media and treated with MMI; lane 3 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 6H medium and not treated with MMI; lane 4 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of the 5H medium; lane 5 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and treated with MMI; lane 6 contains extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and thyroid stimulating hormone (TSH). Lanes a-d in Figure 12C shows the formation of the A complex in the gel shift mobility assays of the 151 radiolabelled fragment (bases 54 to 220 of SEQ ID NO:1) plus FRTL-5 cell extracts can be competed by unlabelled 105 (bases 588 to 692 of SEQ ID NO:1), 140 (bases 321 to 455 of SEQ ID NO:1), 151 (bases 54 to 220 of SEQ ID NO:1) and 114 (bases 221 to 320 of SEQ ID NO:1) fragments. The incubation mixture in lane (a) contains the 151 radiolabelled fragment (bases to 54 to 220 of SEQ ID NO:1), cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium, and unlabelled 105 fragment (bases to 588 to 692 of SEQ ID NO:1); lane (b) contains the 151 radiolabelled fragment (bases to 54 to 220 of SEQ ID NO:1), cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium, and unlabelled 140 fragment (bases 321 to 455 of SEQ ID NO:1); lane (c) contains the 151 radiolabelled fragment (bases to 54 to 220 of SEQ ID NO:1), cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium, and unlabelled 151 fragment (bases to 54 to 220 of SEQ ID NO:1); lane (d) contains the

radiolabelled 151 fragment (bases 54 to 220 of SEQ ID NO:1), cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and unlabelled 114 fragment (bases 221 to 320 of SEQ ID NO:1).

12D. Shows the gel mobility shift assays of the radiolabelled-140 fragment (bases to 321 to 455 of SEQ ID NO:1) with extracts from treated and untreated FRTL-5 cell maintained in 3H medium. Unlike 5H medium, 3H medium contains no insulin. The incubation in lane (j) contains the 140 radiolabelled fragment (bases 321 to 455 of SEQ ID NO:1) alone; lane (e) contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 3H medium; lane (f) contains cell extracts from FRTL-5 rat thyroid cells maintained in 3H medium and treated with MMI; lane (g) contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 3H medium and treated with TSH; lane (h) contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 3H medium and treated with MMI and TSH; lane (i) contains unlabelled 105 fragment together with cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 3H medium.

Figure 13 shows transfection data with chloramphenicol acetyltransferase (CAT) chimeras showing that MMI inhibits full length MHC Class I PD1 promoter activity. FRTL-5 rat thyroid cells were transfected with the full length PD1 promoter, CAT chimeric construct and the cells either received no treatments ( ), treatment with MMI ( ), treatment with TSH ( ) or treatment with TSH and MMI ( ).

Figures 14A-B shows the gel shift mobility assays of the radiolabelled 238 fragment (bases 456 to 692 of SEQ ID NO:1) (Figure 14(A)) or the radiolabelled K oligonucleotide (SEQ ID NO:38) (Figure 14(B)) with extracts from treated or untreated FRTL-5 rat thyroid cells maintained in 5H medium. The complex affected by MMI is denoted by A.

14A. Shows the gel mobility shift assays of the radiolabelled 238 fragment (bases to 456 to 692 of SEQ ID NO:1) incubated with extracts from treated or untreated rat thyroid FRTL-5 cells maintained in 5H medium and with unlabelled double-stranded (ds) oligonucleotides shown in Figure 10. The incubation in lane 1 contains the 238 radiolabelled fragment (bases to 456 to 692 of SEQ ID NO:1) alone; lane 2 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and not treated with MMI; lane 3 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and unlabelled 105 fragment (bases to 588 to 692 of SEQ ID NO:1); lane 4 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of the 5H medium and unlabelled 114 fragment (bases to 221 to 320 of SEQ ID NO:1); lane 5 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and unlabelled 140 fragment (bases to 321 to 455 of SEQ ID NO:1); lane 6 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and unlabelled 151 fragment (bases to 54 to 220 of SEQ ID NO:1); lane 7 contains cell extracts from FRTL-5 rat thyroid cells maintained in 5H medium and unlabelled K-oligonucleotide (SEQ ID NO:38); lane 8 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and unlabelled ds-oligonucleotide S2 (SEQ ID NO:4) (shown in Figure 10); lane 9 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and unlabelled ds-oligonucleotide S3 (SEQ ID NO:10) (shown in Figure 10); lane 10 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and unlabelled ds-oligonucleotide S8 (SEQ ID NO:8) (shown in Figure 10); lane 11 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and unlabelled ds-oligonucleotide S6 (SEQ ID NO:6) (shown in Figure 10); lane 12 contains cell

extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and unlabelled ds-oligonucleotide S1 (SEQ ID NO:3) (shown in Figure 10); lane 13 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and unlabelled ds-oligonucleotide S7 (SEQ ID NO:7) (shown in Figure 10); lane 14 contains cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and treated with MMI and TSH; lane 15 contains unlabelled K-oligonucleotide (SEQ ID NO:38) plus cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and treated with MMI and TSH.

14B. Shows the gel mobility shift assays of radiolabelled K-oligonucleotide (SEQ ID NO:38) incubated with extracts from treated or untreated rat thyroid cells maintained in 5H medium. In lane 16, the incubation contains the radiolabelled K oligonucleotide (SEQ ID NO:38) with cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium alone. In lane 17 the incubation contains radiolabelled K oligonucleotide (SEQ ID NO:38) with cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and treated with MMI; lane 18 contains the radiolabelled K oligonucleotide (SEQ ID NO:38) with cell extracts from FRTL-5 rat thyroid cells maintained in the presence of the 5H medium and treated with TSH; lane 19 contains the radiolabelled K oligonucleotide (SEQ ID NO:38) and cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium and treated with MMI and TSH.

Figures 15A-15B show the effect of MMI and TSH on the transcription rate of MHC class I in FRTL-5 thyroid cells maintained in medium with insulin and 5% serum (Figure 15A) or without insulin and only 0.2% serum (Figure 15B). FRTL-5 thyroid cells maintained for 7 days in 5H medium plus 5% calf serum (Figure 15A) or 4H medium plus 0.2% serum (Figure 15B) were exposed to $1x10^{-10}$M TSH,

5 mM MMI, or both. After 24 hours, nuclei were isolated and incubated with [^{32}P]UTP before nuclear RNA was purified, then hybridized to an excess of class I cDNA and β-actin (Saji, M., Moriarty, et al., (1992b) J. Clin. Endocrinol. Metab. 75, 871-878; Isozaki, O., et al., (1989) Mol. Endocrinol. 3, 1681-1692). After autoradiography and densitometry, the ratio of radiolabeled class I to actin RNA was set at unity in the control cells never exposed to TSH or MMI and the values from treated cells compared. The level of ß-actin RNA transcripts was not affected by the treatments. Values are the mean of 3 experiments; significant increases or decreases at $P<0.05$ (*) or $P<0.01$ (**) are noted. The class I transcription rate in control cells in 4H medium plus 0.2% serum (Figure 15B) was approximately 3.2-fold higher than in 5H medium plus 5% serum (Figure 15A), consistent with an approximately 4-fold higher in class I RNA levels under the respective conditions (Saji, M., Moriarty, et al., (1992b) J. Clin. Endocrinol. Metab. 75, 871-878).

Figures 16A-16B show the effect of MMI and TSH on the promoter activity of CAT chimeras of 5'-deletion mutants of the swine class I promoter in FRTL-5 cells. (Figure 15A) FRTL-5 cells grown in 6H medium (+TSH) were transfected by electroporation with the different constructs of the PD1 5'-flanking region. After 12 hours, the medium was changed to fresh 6H medium (+TSH), fresh 6H medium plus 5 mM MMI (+TSH/+MMI), or fresh 5H medium with no TSH or MMI; CAT activity was measured 36 hours later. Conversion rates were normalized to luciferase levels and protein; the activity of the -1100 bp construct in cells maintained in 6 H medium (first black bar) was assigned a control value of 100%. Differences in the basal level of expression for the different constructs reflect activity of different regulatory elements which have already been described Weissman, J. D. and Singer, D. S. (1991) Mol.

Cell. Biol. 11, 4217-4227; Giuliani, C., et al., (1995) J. Biol. Chem. 270, 11453-11462; Ehrlich, R., et al., (1988) Mol. Cell Biol. 8, 695-703; Maguire, J. E., et al., (1992) Mol. Cell. Biol. 12, 3078-3086; Howcroft, T. K., et al., (1993) EMBO J. 12, 3163-3169), some of which are summarized in (Figure 16B). Values are the mean ± S.E. of three different experiments, each performed in duplicate. (Figure 15B) Graphic representation of the different constructs. Regulatory elements noted include the following: (a) the silencer/enhancer region important in regulating constitutive class I levels in different tissues; (b) Enhancer A; (c) the interferon response element; (d) the 38 bp constitutive silencer containing (e) the CRE-like sequence within the constitutive silencer element. Also noted is (f) the CCAAT box important in initiation of transcription. The numbering of these elements and in all subsequent figures is determined from +1, the start of transcription (Giuliani, C., et al., (1995) J. Biol. Chem. 270, 11453-11462) which is nucleotide 1091 in Figure 9.

Figures 17A-17B show the ability of oligonucleotides to prevent formation of the Fr140 protein/DNA complex which is modulated by MMI and TSH (Figure 12A). (Figure 17B) indicates it is the silencer element, -724 to -697 bp which is involved in complex formation (Figure 17A); formation is also inhibited by class I promoter fragments containing related silencer element sequences 5' and 3' to Fr140 two of which are described in Figure 11. In Figure 17A, cells were cultured in 5H medium with no TSH for 7 days. Cell extracts were prepared and incubated with the Fr140 radiolabeled probe, -770 to -636 bp, of the PD1 promoter. The arrow marked (A) denotes the protein/DNA complex decreased by MMI and TSH and identified as the silencer element based on inhibition by a 250-fold excess of the S2 (SEQ ID No: 4) and S6 (SEQ ID No: 6) but not the E9

oligonucleotides (Fig. 10). The arrow marked (b) denotes the protein/DNA complex identified as the enhancer element based on inhibition by the S6 (SEQ ID No: 6) oligonucleotide, which only partially inhibits the silencer element, and E-9 which inhibits only the enhancer. The S3 (SEQ ID No: 10) oligonucleotide is the control and has no effect. The arrow denoted (c) indicates a protein/DNA complex whose function is unknown at this time. In Figure 17B, the same extracts were incubated with a 100-fold excess of unlabeled Fragments 5' and 3' to Fr140: Fr 151 (-1047 to -881 bp), Fr114 (-880 to -771- bp), and Fr105 (-503 to -399 bp). These class I regions contain areas with significant homology to the silencer in Fr140 (SEQ ID No: 37) (Figs. 11 and 12A-D).

Figures 18A-18C show the effect of increasing salt concentration (Figure 18A) and of antisera to the p50 or p65 subunits of NF-κB (Figure 18B) or to c-fos family members (Figure 18C) on the silencer complex formed with Fr140 (SEQ ID No: 37). FRTL-5 cells were cultured in 5H medium with no TSH for 7 days. Cell extracts were prepared, incubated with the Fr140 (SEQ ID No: 37) radiolabeled probe, -770 to -636 bp, of the PD1 promoter, and complex formation measured by EMSA. The silencer complex characterized in Figures 12A-D, 14A-B, and 17A-B is denoted with an arrow. In (Figure 18A), increasing concentrations of KCl were added to the incubation mixture; in (Figure 18B) and (Figure 18C) the noted antisera or normal serum (lane 3, Figure 18C) were preincubated with the extracts before probe was added. The dashed line denotes the location of a "supershifted" complex. Antisera against fos B, c-fos, fra-1, and fra-2 are, respectively, sc-48, sc-413, sc-183, and sc-57 from Santa Cruz. The results indicate the silencer complex (arrow) is comprised of more than one protein/DNA complex (Fig. 18A-C) and that one of the proteins is the p65 subunit of NF-κB (Fig. 18B) and another is a c-fos family

member (Fig. 18C), probably other than fra-1, fra-2 or fos B.

Figures 19A-19C show the CAT activity of p(-1100)CAT (Figure 19A), p(-127)CAT (Figure 19B), or p(-127NP)CAT (Figure 19C) transfected into FRTL-5 cells with or without cotransfection by a plasmid containing an oligonucleotide with the sequence of oligo K (SEQ ID No: 38), the thyroglobulin (TG), insulin response element (IRE), or oligo KM, a mutant thereof which loses insulin-responsiveness (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Aza-Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306). FRTL-5 cells grown in 5H medium plus 5% calf serum were cotransfected with the different constructs of the PD1 5'-flanking region plus 20 or 40 μg of a plasmid with an oligonucleotide having the sequence of oligo K (Oligo K1, and Oligo K2, respectively). Alternatively, cotransfection was with 40 μg of oligo KM, a mutated form of oligo K described previously (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Aza-Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306) p(-127NP)CAT is the p(-127)CAT chimera (Figure 16A) containing a nonpalindromic mutation (see Fig. 25A) of the CRE in the downstream silencer. CAT activity was measured 36 hours later and conversion rates normalized to growth hormone levels. The activity of control transfections with the vector into which the oligo K sequences were inserted was assigned a value of 100% (first open bar in each panel). Differences in the CAT activity of cells cotransfected with Oligo K (SEQ ID No: 38) or its mutant were compared to the control values. Values are the mean ± S.E. of three different experiments, each performed in duplicate. In (Figure 19A), one star(*) denotes a significant ($P<0.05$) increase in activity caused by oligo K1; two stars (**) denotes a significant increase by oligo K2

(P<0.01). In (Figure 19C), the increase in the presence of oligo K1 is significant, P<0.01.

Figures 20A-20B shows the nucleotide and deduced amino acid sequence of the clone designated Sox-4 obtained by screening a rat FRTL-5 cell expression library with oligonucleotide K (SEQ ID No: 38), the insulin responsive element of the thyroglobulin promoter, which can inhibit the ability of methimazole (MMI) and TSH to decrease the silencer complex with Fr140 of the class I promoter (see Figure 12 A-D and 14A-B). The sequence contains 1422 nucleotides whose open reading frame encodes a 442 amino acid residue protein with a molecular weight of about 53,040. Differences in amino acid residues from mouse Sox-4 are noted under the rat sequence by the specific replacement residues. Differences from human Sox-4 (Van de Wetering, M., et al., (1993) EMBO Journal 12,3847-3854) are noted by dashed lines. Rat and mouse Sox-4 are 32 residues smaller than human Sox-4 (Farr, C. J., et al., (1993) Mammalian Genome 4, 577-584). All of the extra residues in human Sox-4, which are not noted, cluster within the one region of the protein containing the amino acid differences from mouse and human Sox-4 as noted; they are in large measure glycine and alanine residues (Farr, C. J., et al., (1993) Mammalian Genome 4, 577-584). The boxed residues are amino acids which are the same in rat, mouse and human Sox-4 genes. The Sox-4 proteins are members of the HMG (high mobility group) class of transcriptional regulators, which bind DNA in a sequence specific fashion; the HMG box is boxed in bold. In addition to the HMG box, a common feature of all three Sox-4 proteins is a serine-rich carboxy terminal tail with multiple putative casein kinase and histone kinase phosphorylation sites (Van de Wetering, M., et al., (1993) EMBO Journal 12,3847-3854; Farr, C. J., et al., (1993) Mammalian Genome 4, 577-584).

Figure 21 shows the ability of recombinant rat Sox-4 protein, 50 ng, to form a protein-DNA complex when incubated with radiolabeled oligonucleotide K (SEQ ID NO:38), oligonucleotide Z (the equivalent insulin responsive element of the thyroid peroxidase promoter), or mutants thereof, which lose the ability to compete for the binding of thyroid transcription factor-2 (TTF-2) in EMSA (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Francis-Lang, H., et al., (1992) Mol. Cell Biol. 12, 576-588; Aza Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306). Also used is a control oligonucleotide, oligonucleotide C from the thyroglobulin promoter, which is adjacent to the oligonucleotide K site and does not bind TTF-2 but can bind thyroid transcription factor-1 (TTF-1) or Pax-8 (Guazzi, S., et al., (1990) EMBO J., 9, 3631-3639; Francis-Lang, H., et al., (1992) Mol. Cell Biol. 12, 576-588; Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Zannini, M., et al., (1992) Mol. Cell Biol. 12, 4230-4241; Aza Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306). The synthetic double-stranded oligonucleotides were end labeled with [γ^{32}P]ATP and T4 polynucleotide kinase then purified on an 8% native polyacrylamide gel before use. Binding reactions were carried out in a volume of 30 μl for 20 min at room temperature; reaction mixtures contained 1 μg recombinant protein and 0.5 μg poly(dI-dC) in 10 mM Tris-Cl (pH 7.9), 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ethylenediamine tetraacetic acid (EDTA), 5% glycerol, and 200 mM Kcl. Labeled probe, 50,000 cpm, was added and the incubation continued an additional 20 min at room remperature. DNA-protein complexes were separated on 5% native polyacrylamide gels.

Figures 22A-22B show Northern analyses of different rat tissues (Figure 22A) and of rat FRTL-5 thyroid cells treated with various hormone mixtures for the periods of time noted, 24 hours or 1 week (Figure

22B). In Figure 21 A, a rat Multiple Tissue Northern Blot (Clontech, Palo Alto, CA) was employed for the Northern analysis, containing mRNA from the noted rat tissues. In Figure 21B, the mRNA was prepared from cultured nonfunctional FRT (Fisher rat thyroid), BRL (Buffalo rat liver), and functional FRTL-5 (Fisher rat thyroid strain L-5) cells using the QD™ rapid poly (A)⁺mRNA isolation system (5Prime→3Prime Inc., Boulder, CO) or from human thyroid or thymus tissue and rat ovary tissue. Northern analyses used 1.5 μg RNA per lane, 1% agarose gels containing 2% formaldehyde, and nylon filters (Nytran, Schleicher and Schuell, Keene, NH). For both Figures 21A and 21B, full length rat SOX-4 or rat ß-actin cDNA (provided by Dr. B Paterson, NCI, Bethesda, MD) was radiolabeled by random priming. Prehybridization and hybridization ($1.0x10^6$ cpm/ml) was performed in Quickhyb Hybridization Solution (Stratagene, LaJolla, CA). Washings were carried out as previously described (Isozaki, O., et al., (1989) Mol. Endocrinol. 3,1681-1692).

Figure 23 shows the ability of an antibody to Sox 4 to inhibit silencer and enhancer complex formation between Fr140 and an FRTL-5 cell extract. FRTL-5 cells were cultured in 5H medium with no TSH for 7 days. Cell extracts were prepared, incubated with the Fr140 radiolabeled probe, -770 to -636 bp, of the PD1 promoter, and complex formation measured by EMSA. The silencer and enhancer complexes characterized in Figures 12, 14, 17 and 18 are noted. The IgG fraction of sera from preimmune or immunized rabbits (Lanes 2 and 3, respectively) were preincubated with the extracts before probe was added. The antibody was created by immunizing rabbits with KLH-conjugated peptide 359 to 373 (GSSSSDDEDDLLD) of rat Sox-4 (Figure 20) according to a standard protocol (Genosys Biotechnologies, Inc., The Woodlands, Texas). IgG was purified by affinity chromatography with Protein A-Sepharose CL-4B columns and was desalted on a Pierce

(Rockford, IL) desalting column equilibrated in phosphate buffered saline, pH 7.4. The IgG eluent was dialyzed against 100 volumes of phosphate buffered saline, pH 7.4, for 18 hours at 4°C and concentrated by centrifugation at 500xg for 3.5 hours at 4°C in a Centricon 10 unit (Amicon, Beverly MA). IgG could be stored at -20°C until assay. The rabbit antibody used herein reacts with the synthetic peptide at a 1:10,000 dilution, is peptide specific, i.e. does not recognize another hydrophilic peptide from Sox-4 mimicking residues 226 to 239 (Figure 20), and can detect Western blotted recombinant Sox-4 as measured by ELISA.

Figures 24A-24B show DNAase I protection analysis on a class I PD-1 genomic fragment, -800 to -605 bp, created by polymerase chain reaction (PCR) (Saiki, R. K., et al., (1988) Science 239, 487-491 46). The template was the PD-1 5'-flanking region containing the 140 fragment (Ehrlich, R., et al., (1988) Mol. Cell Biol. 8, 695-703; Maguire, J., et al., (1992) Mol. Cell Biol. 12, 3078-3086). The forward primer contained a BamHI site on the 5'-end (underlined), ATAGGATCCGAATAGGAAACACGGAGTATACTG ATTCAG, and extended from -800 to -770 bp of the PD-1 sequence. The reverse primer contained a HindIII site (underlined), ATAAAGCTTCACTGGAGGTTTATGTCTGCTTCTGTGCTG and extended from -605 to -634 bp. The fragment was digested by BamHI and HINDIII and inserted into a CAT chimera as described (Ehrlich, R., et al., (1988) Mol. Cell Biol. 8, 695-703; Maguire, J., et al., (1992) Mol. Cell Biol. 12, 3078-3086; Giuliani, C., et al. (1995) J. Biol. Chem. 270, 11453-11462). As needed, it was isolated by restriction enzyme digestion (Ehrlich, R., et al., (1988) Mol. Cell Biol. 8, 695-703; Maguire, J., et al., (1992) Mol. Cell Biol. 12, 3078-3086); and purified from 2 % agarose gels using a QIAEX extraction kit (Quiagen, Chatsworth, CA). Coding strand data are presented in Figure 24A; noncoding strand results are in Figure 24B. The fragment was end-labeled with [α^{32}P]dCTP and Klenow fragment, then purified

on an 8% native polyacrylamide gel. DNAase I footprinting (Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al., (1993) J. Biol. Chem. 268, 24125-24137) used 1, 5 or to 10 μg purified recombinant proteins. Initial incubation was for 20 min at room temperature in a 20 μl reaction volume with 10 mM Tris-Cl, pH7.6, 5 mM $MgCl_2$, 0.1% triton X-100, no KCl, and 1 μg poly(dI-dC). After addition of the probe (50,000 cpm), the reaction mixture was incubated for 20 min at room temperature. DNA probes were then digested with 0.5 unit DNAase I (Promega, Madison, WI) for 1 min at room temperature. The reactions were terminated with 80 μl stopping solution (20 mM Tris HCl, pH 8.0, 250 mM NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS), 10 μg proteinase K, and 4 μg sonicated calf thymus DNA). After incubation at 37 C for 15 min, the digested products were phenol extracted, ethanol precipitated, and separated on an 8% sequencing gel. Maxam and Gilbert A+G and C+T sequencing reactions (Maxam, A. M., et al., (1980) Methods Enzymol. 65, 499-560) were used to locate the footprinted regions. The region footprinted by Sox-4 and a control recombinant protein, the p50 subunit of NF-κB, are noted to the right of each panel and compared with the silencer and enhancer regions of the class I promoter (see Figure 10).

Figures 25A-25B show the effect of modifications of the CRE-like element on activity of the p(-127)CAT promoter and effect of CRE-like element on the activity of a heterologous promoter. In Figure 25A, the CRE-like element in the p(-127)CAT promoter construct was either deleted (ΔCRE) or mutated to a nonpalindromic sequence as noted (NP CRE). The CAT activities of these derivative constructs were compared with that of the parental p(-127)CAT activity following transfection into FRTL-5 cells and their incubation in 3H medium plus 5% calf serum for 2 days. Conversion rates were normalized to hGH

levels and protein. CAT activities are expressed relative to the parental p(-127)CAT, which averaged 2-fold higher CAT activity than the pSV0 control chimera; data are the mean ± S.E. for 3 separate experiments. Statistically significant increases ($P<0.05$) from p(-127)CAT are noted by the star. In Figure 25B, class I DNA sequences between -127 and -90 bp (designated CRE) were introduced at the 3' end of constructs containing the SV40 promoter ligated to the CAT gene and transfected into FRTL-5 cells as described (Shimura, Y. et al. (1994) J. Biol. Chem. 269, 31908-31914). CAT activities were measured after maintaining the cells for 2 days in 3H medium plus 5% calf serum and normalizing conversion rates to hGH levels and protein. Constructs containing the CRE are diagrammatically represented on the left of the Figure. Arrows depict the orientation and the number of copies of the CRE present. Also shown are two mutant constructs from which the CRE site was either deleted (ΔCRE) or mutated to a nonpalindromic (NP CRE) form (Fig. 25A). CAT activities are presented relative to the parental promoter construct, pCAT, which contains a minimal SV40 promoter and averaged 44 ± 5%; values are the mean ± S.E. for 3 separate experiments. Statistically significant decreases are indicated as $P< 0.05$ (*) or $P<0.01$ (**).

Figures 26A-26B show that TSH treatment of FRTL-5 cells induces the formation of novel protein/DNA complexes between cell extracts and a fragment of the 5'-flanking region of the class I promoter from -168 to -1 bp (Fr168; nucleotides 923 to 1190 in Figure 9). Figure 26A, is a diagrammatic representation of the 5' flanking region of the class I gene promoter. All numbers are relative to the start of transcription, designated +1 as defined in Giuliani C., et al (1995) J. Biol Chem. 270:11453-11462 herein incorporated by reference. The CRE-like sequence is indicated at -107 to -100 base pairs (bp)., as are the positions of the CAAT and TATA boxes, enhancer A (EnH),

and the interferon response element (IRE). The DNA fragments used in the electrophoretic mobility shift assays (EMSA) are indicated. In Figure 26B, EMSA are performed using the radiolabeled Fr168 probe incubated with extracts from FRTL-5 cells maintained in the absence (-) or presence (+) of $1x10^{-10}$M TSH for 48 hours after 6 days in 3H medium plus 5% serum. The Fr168 probe was incubated with either extract alone (lanes 1 and 5) or in the presence of a 100-fold excess of either unlabeled Fr168 (lanes 2 and 6), Fr127 (nucleotides 964 to 1090 in Figure 9), (lanes 3 and 7), or CRE-1, a 38 bp silencer including the CRE like site (nucleotides 964 to 1001 in Figure 9) (lanes 4 and 8). In this and all other experiments involving CRE-1 to be described it can be replaced by a 48 base pair (bp) sequence extending 10 nucleotides on the 3' end with no difference on results. Protein/DNA complexes are denoted by the letters A to G; F and G represent complexes present in the TSH-treated cell extracts only.

Figures 27A-27B show the effect of MMI and/or TSH treatment of FRTL-5 cells on the ability of cellular extracts to form protein/DNA complexes with radiolabeled Fr168 of the MHC 5'-flanking region (Figure 27A) in the absence or presence of an unlabeled oligonucleotide (oligo TIF) with the sequence of the TSH receptor (TSHR) insulin response element (Figure 28C; Shimura, Y., et al. (1994) J. Biol. Chem. 269, 31908-31914) or (Figure 27B) an unlabeled oligonucleotide with the sequence of the 38 bp downstream silencer with (NP CRE) or without (CRE-1) a nonpalindromic mutation of the CRE. Cells were maintained 7 days in 5H medium (no TSH) plus 5 % calf serum (lane 1; 5H Basal) at which time 5 mM MMI (lane 2), $1x10^{-10}$M TSH (lane 5), or the two together (Lane 4) were added for 36 hours before extracts were prepared and incubated with ^{32}P-radiolabeled Fr168 of the MHC 5'-flanking region, -168 bp to +1. In Figure 27A, complex formation was evaluated

by EMSA in the absence of an unlabeled competitor (lanes 1, 2, 4, 5) or in the presence of a 250-fold excess of an oligonucleotide with the sequence of the TSHR insulin response element (Oligo TIF) or the TG insulin response element (Oligo K). In Figure 27B, complex formation with the extract from MMI-treated cells was evaluated in the absence of an unlabeled competitor (lane 2) or in the presence of a 250-fold excess of an oligonucleotide with the sequence of the downstream 38 bp silencer (CRE-1; lane 3) or its nonpalindromic counterpart (NP CRE as noted in Fig. 25A; lane 4). The probe alone is in lane 1 of Figure 27B. The arrow denotes the complex whose formation is increased by TSH/MMI treatment of the cells but inhibited from forming by in vitro addition of oligo TIF or CRE-1.

Figures 28A-28C show the effect of MMI on the promoter activity of p(-1100)CAT or p(-127)CAT transfected into FRTL-5 cells with or without cotransfection by a plasmid containing an oligonucleotide having a mutated sequence of the TSHR insulin response element (Shimura, Y., et al. (1994) J. Biol. Chem. 269:31908-1914) (TIF mutant 2) which does not lose insulin-responsiveness. Using a DEAE-dextran procedure, FRTL-5 cells grown in 6H medium (+TSH) were cotransfected with the different constructs of the PD1 5'-flanking region plus a plasmid with or without an oligonucleotide having the sequence of mutant 2 of oligo TIF, the TSHR insulin response element (Shimura, Y., et al., (1994) J. Biol. Chem. 269, 31908-31914). After 12 hours, the medium was changed to fresh 6H medium plus or minus 5 mM MMI (MMI) and CAT activity was measured 36 hours later. After normalization of conversion rates and protein values, the activity of the -1100 (Figure 28A) or 127 bp (Figure 28B) constructs in cells maintained in 6 H medium and cotransfected with plasmid containing no oligonucleotide was assigned a control value of 100% (first open bar in each panel). Differences in the expression of cells cotransfected with

the oligonucleotide containing the oligo TIF mutant (second open bar in each panel) were evaluated both in the absence (open bars) or presence of MMI (stippled bars). In Figure 28C, the sequences and activities of the mutants are summarized. Oligo TIF mutant 2 contains a mutation which loses single strand binding activity but retains insulin responsiveness when compared with wild type TSHR (Figure 28C; Shimura, Y., et al., (1994) J. Biol. Chem. 269, 31908-31914). Values are the mean ± S.E. of three different experiments, each performed in duplicate. In Figures 28A and 28B, one star (*) denotes a significant decrease ($P<0.01$) in activity caused by MMI; three stars (***) denotes a significant loss ($P<0.01$) in the ability of MMI to decrease promoter activity. In Figure 28A, two stars (**) denotes a an increase ($P<0.05$) in basal promoter activity caused by the oligonucleotide cotransfection in the absence of MMI.

Figures 29A-29B show that in the absence of TSH, the 38 bp silencer region containing the CRE-like sequence, -127 to -90bp forms multiple protein/DNA complexes with extracts from FRTL-5 cells, one of which appears to be CREB. Figure 29A additionally shows their formation depends on the CRE-like sequence, -107 to -100 bp, and on sequences flanking the CRE. The radiolabeled double-stranded 38 bp DNA fragment, -127 to -90 bp, termed CRE-1, was incubated with extracts from FRTL-5 cells maintained in 3H medium plus 5% calf serum for 6 days and complexes were analyzed by EMSA. In Figure 26A, complex formation was evaluated in the presence or absence of the noted unlabeled double-stranded oligonucleotides: CRE-1, ΔCRE-1 with the CRE-like sequence deleted, and a Promega CRE which contains the consensus CRE and flanking residues from the somatostatin promoter. The amount of each competitor in fold excess over probe is noted at the top of each set of gels, along with a diagrammatic representation of the structure of the competitor. In

Figure 26B, incubations were performed in the presence of 2 ul of rabbit antiserum against CREB2, mXBP, ATF2, or CREB-327, as indicated (lanes 1-4, respectively); the dashed line notes the supershifted complex resultant from incubation with the CREB-327 antibody. Letters represent groups of complexes formed by the extract. The improved separation of the A complex region in the Experiment in Figure 26B was achieved using a lower gel concentration during the separation.

Figures 30A-30C shows that the 38 bp silencer region containing the CRE-like sequence forms complexes with both thyroid transcription factor-1 (TTF-1) and Pax-8 in addition to CREB; it further shows their formation is independent of the poly(dI-dC) concentration. The double-stranded, radiolabeled 38 bp DNA fragment, -127 to -90 bp (CRE-1), was incubated with extracts from FRTL-5 cells maintained in 5H medium plus 5% calf serum for 6 days; complexes were analyzed by EMSA in 3.0 (Figure 30B) as well as 0.5 (Figure 30A) μg/ml poly(dI-dC). In Figure 30A, complex formation was evaluated in the presence or absence of the noted unlabeled, double-stranded oligonucleotides: CRE-1, ΔCRE-1 with the CRE-like sequence deleted, an oligonucleotide containing the TTF-1 element in the TSHR, and a mutant thereof which loses TTF-1 binding and activity in the FRTL-5 cell. The sequences of the oligonucleotides containing the TSHR TTF-1 element and its mutant are presented in the Figure 30C (Shimura, H., et al., (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al., (1995) Endocrinology, 136, 269-282). The TSHR TTF-1 site does not bind Pax-8 (Shimura, H., et al., (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al., (1995) Endocrinology, 136, 269-282; Civitareale, D., et al., (1993) Mol. Endocrinol. 7, 1589-1595). In Figure 30B complex formation was again evaluated in the presence or absence of CRE-1, an oligonucleotide containing the TTF-1 element in the TSHR, and a mutant thereof, which loses

TTF-1 binding and activity in the FRTL-5 cell. In addition, the incubations were performed in the presence of a double-stranded oligonucleotide from the thyroglobulin promoter which contains a site able to interact with TTF-1 and Pax-8, termed TG oligo C, and a mutant thereof, which loses TTF-1 and Pax-8 binding and activity in FRTL-5 cells. The sequences of the oligonucleotides containing the TG oligo C element and its mutant are presented in the Figure 30C; (Civitareale, D., et al., (1989) EMBO J., 2537-2542; Guazzi, S., et al., (1990) EMBO J. 9, 631-3639; Francis-Lang, H., et al., (1992) Mol. Cell. Biol. 12, 576-588; Zannini, M., et al., (1992) Mol. Cell. Biol. 12, 4230-4241; Shimura, H., et al., (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al., (1995) Endocrinology, 136, 269-282). The amount of each competitor was 100-fold in excess of probe. Letters represent groups of complexes formed by the extract; the TTF-1 and Pax-8 containing complexes with the 38 bp silencer are noted based on the inhibition data.

Figures 31A-31C show the C complexes formed with the 38 bp silencer region containing the CRE-like sequence appear to involve proteins able to bind either its coding or noncoding strands (Figure 31A); in addition these show that these appear to involve single strand binding proteins which are important in TSH/cAMP suppression of TSHR gene expression in FRTL-5 thyroid cells. (Figure 31B). The double-stranded radiolabeled 38 bp DNA fragment, -127 to -90 bp, termed CRE-1, was incubated with extracts from FRTL-5 cells maintained in 5H medium plus 5% calf serum for 6 days; complexes were analyzed by EMSA in 0.5 μg/ml poly(dI-dC). In (Figure 31A) complex formation was evaluated in the presence or absence of a 100-fold excess over probe of the unlabeled single strand oligonucleotides comprising the coding and noncoding strand of CRE-1. In (Figure 31B) complex formation was evaluated in the presence or absence of a single strand

oligonucleotide from the noncoding strand of the TSHR, which binds a single strand binding protein termed SSBP (Shimura, H., et. al. (1995) Mol. Endocrinol., 9, 527-539), and a single strand oligonucleotide from the coding strand of the TSHR which binds a Y-box protein termed thyrotropin receptor suppressor element protein-1 (TSEP-1) (See Figure 38 and Example 11). These are termed oligo SSBP and oligo TSEP-1, respectively; their sequences are presented in Figures 31C. The amount of each unlabeled oligonucleotide, in fold-excess over probe, is noted. Cell extracts were made by a modification of a described method (Dignam, J., et al., (1983) Nucleic Acids Res. 11, 1475-1489). The reaction mixtures contained 1.5 fmol of [^{32}P]DNA, 3 μg cell extract, and 0.5 μg poly(dI-dC) in 10 mM Tris-Cl (pH 7.9), 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ethylenediamine tetraacetic acid and 5% glycerol. Unlabeled double- or single-stranded oligonucleotides were also added to the binding reaction as competitors and incubated with the extract for 20 min prior the addition of labeled DNA. Following incubations, reaction mixes were subjected to electrophoresis on 4 or 5 % native polyacrylamide gels at 160 V in 1xTBE at 4°C.

Figures 32A-32B show that the single strand components of the 38 bp silencer region containing the CRE-like sequence (CRE-1) form complexes with proteins in FRTL-5 thyrocyte extracts which bind the Y-box or TSHR suppressor element protein-1 (TSEP-1) (Figure 32A) and the single strand binding protein (SSBP) (Figure 32B) sites of the TSHR minimal promoter. The single-strand components of the radiolabeled 38 bp DNA fragments, -127 to -90 bp, termed CRE-1, were incubated with extracts from FRTL-5 cells maintained in 5H medium plus 5% calf serum for 6 days; complexes were analyzed by EMSA in 0.5 μg/ml poly(dI-dC) as described in Figure 30A-C. In Figure 32A, complex formation was evaluated using the radiolabeled coding strand of CRE-1 in the presence or absence of a

100-fold excess over probe of the unlabeled single strand oligonucleotides containing wild type (WT) and mutated sequences of each of the three TSEP-1 binding sites of the TSHR (see Example 11). The sequences of the competitor oligonucleotides and their location in the TSHR 5'-flanking region are noted below the gel; the dark bars represent the CCTC motif which appears to be important for TSEP-1 binding by each site in the TSHR (See Example 11). In each case the mutant 1 (Mut. 1) oligonucleotide binds TSEP-1 whereas the mutant 2 (Mut. 2) form loses binding activity (See Example 11). The oligo TSEP-1 site accounts for the 5' decanucleotide activity in the tandem repeat (TR) of the TSHR which is known to suppress the constitutive enhancer activity of the TSHR CRE (Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al., (1993) J. Biol. Chem. 268, 24125-24137); the TR and CRE sites of the TSHR 5'-flanking region are noted. In (Figure 32B), complex formation was evaluated using the radiolabeled noncoding strand of CRE-1 in the presence or absence of a 100-fold excess over probe of the unlabeled single strand oligonucleotide containing wild type (WT) and a mutated sequence of the SSBP binding site on the noncoding strand of the TSHR 5'-flanking region. The SSBP binds to a site on the noncoding strand of the TSHR 5' and contiguous with the TTF-1 site, which is double-stranded; the mutation noted eliminates SSBP binding and activity but not TTF-1 binding and activity (Shimura, H., et al., (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al., (1995) Endocrinology. 136, 269-282; Shimura, H., et al., (1995) Mol. Endocrinol., 9, 527-539).

Figure 33 shows TSH treatment of FRTL-5 cells decreases CREB and TTF-1 binding to the class I 38 bp silencer region containing the CRE-like sequence and causes a relative increase in C complex formation, which includes protein/DNA complexes with TSEP-1 and the SSBP.

The radiolabeled double-stranded 38 bp DNA fragment, -127 to -90 bp, termed CRE-1, was incubated with extracts from FRTL-5 cells maintained in 5H medium plus 5% calf serum for 6 days then treated for 16 additional hours with the same medium or the same medium plus $1x10^{-10}$M TSH. Incubations were performed in the presence or absence of 2 ul of rabbit antiserum against CREB-327. Complexes were analyzed by EMSA, as described in Figs. 29, 30, 31 and 32, but in the presence of 3 μg/ml poly(dI-dC). The A, B, and C complex areas are noted (Figures 29, 30, 31 and 32); the A region contains complexes with CREB and Pax-8, the B with TTF-1, and the C with TSEP-1 and SSBP (Figs. 29, 30, 31 and 32).

Figures 34A-34B show the effect of oligo TIF (Shimura, Y. et al., (1994) J. Biol. Chem., 269:31908-3194; Figure 32), one of the TSEP-1 binding sites on the TSHR, on the formation of the TSH-induced protein/DNA complexes with radiolabeled Fr168, -168 to +1 bp (Fig. 34A) or radiolabeled Fr127, -127 to +1 bp.(Fig. 34B). FRTL-5 cells were maintained 6 days in 5H medium with 5% calf serum at which time fresh 5H medium or 5H medium containing $1x10^{-10}$M TSH (6H) was added for 36 hours. Cell extracts were prepared, incubated with ^{32}P-radiolabeled Fr168 (Figure 34A) or Fr127 (Figure 34B) of the MHC 5'-flanking region, and evaluated by EMSA. Incubations were additionally performed in the presence or absence of double stranded oligo TIF, a TSEP-1 binding site on the TSHR or mutants thereof (Fig. 32), one of which, TIF Mut-2, loses TSEP-1 binding activity because of a mutation in the CCTC binding motif. In (Figure 34B) we additionally show that the oligonucleotide from the TG promoter able to bind TTF-1 or Pax-8 does not prevent formation of the TSH-induced complex (negative control), whereas CRE-1 does inhibit formation of the TSH-induced complex.

Figures 35A-35C shows the effect of 10 μM forskolin on the Class I gene promoter activity of a

series of deletion mutants spanning 1100 bp of 5' flanking sequence (A) or of the 38 bp silencer region containing the CRE when attached to a heterologous promoter. In (Figure 35A), FRTL-5 cells were grown to 75% confluency then maintained 6 days in 5H medium plus 5% calf serum. Cells were returned to 6H medium for 12 h and transfected with a CAT chimera containing 1100 bp of 5'-flanking region of the swine class I promoter p(-1100)CAT by electroporation or with 5'-deletions of p(-1100)CAT. Each deletion is denoted by the position of its 5' residue. After 12 additional hours the medium was changed to fresh 5H medium in the presence or absence of 10 μM forskolin. CAT activity was assayed 36 hours later; conversion rates were normalized to hGH levels and protein. Results are expressed relative to pSV0, the parental CAT vector with no promoter and no forskolin in the medium, whose activity is set at 100%. Values are the mean ± S.E. of 3 experiments; statistically significant increases or decreases at $P<0.05$ (*) or $P<0.01$ (**) are noted. In (Figure 35B) cells were similarly handled but were transfected with pCAT Promoter containing one or two copies of the 38 bp silencer as illustrated in Figure 35C. After 12 additional hours, the medium was changed to fresh 5H medium in the presence or absence of 10 μM forskolin. CAT activity was assayed 36 hours later; conversion rates were normalized as above, and results expressed relative to the parental pCAT Promoter vector with no insert and no forskolin in the medium, whose activity is set at 100%. Values are the mean ± S.E. of 3 experiments; a statistically significant effect of forskolin at $P<0.05$ (*) is noted. The forskolin action is duplicated in Figure 35B by either $1x10^{-10}$M TSH and 5mM MMI.

Figure 36 shows the formation of the TSH-induced complex depends on DNA sequence elements between -90 and -1 bp. FRTL-5 cells were maintained 6 days in 5H medium with 5% calf serum at which time fresh 5H medium or 5H

medium containing $1x10^{-10}$M TSH (6H) was added for 36 hours. Cell extracts were prepared, incubated with ^{32}P-radiolabeled Fr127 of the MHC 5'-flanking region, and evaluated by EMSA. Incubations were additionally performed in the presence or absence of unlabeled Fr127, Fr89, or CRE-1 (positive control), at the noted fold-concentrations over probe.

Figures 37A-37D show the ability of unlabeled Fr140 to prevent formation of the MMI-induced protein/DNA complex with radiolabeled Fr168 of the MHC 5'-flanking region (Figure 37A) and of oligonucleotide E9, the specific inhibitor of the upstream enhancer (Figure 17A), to prevent complex formation with the 38 bp downstream silencer (Figure 37B). In Figure 37A, FRTL-5 cells were maintained 7 days in 5H medium (no TSH) plus 5 % calf serum, at which time 5 mM MMI (5H MMI+) or 5 mM MMI plus $1x10^{-10}$M TSH (5H MMI/TSH+) were added for 36 hours. Cell extracts were prepared, incubated with radiolabeled Fr168 of the MHC 5'-flanking region, -168 bp to the start of transcription (+1), and complex formation evaluated by EMSA in the absence of an unlabeled competitor (1st and 3rd lanes) or in the presence of a 250-fold excess of unlabeled Fr140 (2nd lane). The arrow denotes the complex whose formation is increased by TSH/MMI treatment of the cells but inhibited from forming by in vitro addition of oligo TIF or CRE-1 (see Figure 34). In (Figure 37B), the extract from FRTL-5 cells maintained 7 days in 5H medium (no TSH) plus 5 % calf serum was incubated with the radiolabeled 38 bp downstream silencer (CRE-1, depicted in Figure 37C). Incubation was in the presence or absence of a 250-fold excess of the noted unlabeled oligonucleotides. The arrows denoted a and b identify, respectively, the TTF-1 and TSEP-1/SSBP complexes with the downstream silencer. Oligonucleotide S6, which inhibits formation of both the silencer and enhancer (Figure 17A) and E9 which inhibits formation of only the enhancer (Figure 17A)

decrease the formation of both complexes as does CRE-1 (lanes 4 and 8 vs. 3 respectively; Figure 37B).

Figures 38A-38B' show the nucleotide and deduced amino acid sequence of TSEP-1 as derived from Clones 9, 31, and 40 obtained by screening a thyroid cell expression library for a suppresor protein reactive with the 5' decanuclotide tandem repeat of the TSHR. Organization of the rat TSEP-1 cDNA and each clone is shown in Figure 38A; the rectangular box indicates the coding region. Nucleotide and amino acid sequence of TSEP-1 are shown in Figure 38B. Nucleotide sequence is numbered from the start codon of TSEP-1 protein. Solid underlining indicates six possible nuclear localization signals. To isolate the cDNA encoding the protein that could bind to the coding sequence of the 5'-decanucleotide in the rat TSHR minimal promoter and which might function as a suppressor by interacting with it (Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al., (1993) J. Biol. Chem. 268, 24125-24137), a rat FRTL-5 cell expression library was screened (Akamizu, T., et al., (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5677-5681) using a Southwestern procedure (Vinson, C. R., et al., (1988) Genes Dev. 2, 801-806) and a radiolabeled synthetic oligonucleotide representing the coding strand of TR2, ssTR2(+), (Shimura, H., et al., (1993) J. Biol. Chem. 268, 24125-24137). TR2 spans -177 to -138 bp of the rat TSHR promoter, contains both the 5'- and 3'-decanucleotides of the tandem repeat (TR), and extends into the CT-rich domain 5' to the TR (Shimura, H., et al., (1993) J. Biol. Chem. 268, 24125-24137). The protein was designated TSHR suppressor element-binding protein-1 or TSEP-1, in accord with its proposed functional role in TSHR gene expression. (Shimura, H. et al. (1993) J. Biol. Chem. 263:24125-24137). Three clones, with overlapping sequences, were identified as candidates for TSEP-1. Clone 40, 1405 bp, encoded a protein with an

open reading frame of 322 amino acids (Figure 38A). Clone 9, 864 bp, contained 117 bp of 5'-flanking region, the ATG start codon, and the N-terminal portion of the open reading frame defined in clone 40 (Figure 38A). Clone 31, 1390 bp, contained a near full length open reading frame and the 3'-noncoding region of clone 40, including the poly (A) signals (Fig. 38A). The cDNA derived from clone 9, fused with the β-galactosidase gene and induced by IPTG in a transformed bacterial host, hybridized very strongly with the coding strand of TR2 [ssTR2(+)], but very weakly with the noncoding strand, [ssTR2(-)] or double-stranded TR2. It did not hybridize with single or double stranded TR1CRE, which spans -153 to -114 bp and encompasses the 3'-decanucleotide of the TR plus the CRE-like sequence, -139 to -132 bp (Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al., (1993) J. Biol. Chem. 268, 24125-24137). Experiments with purified recombinant protein confirm that the cloned cDNA encoded a protein interacting with the coding strand of the 5' decanucleotide of the thyroid receptor (TR). Transfection experiments establish that it is a suppressor.

Figures 39A-39C show the effect of mutations in each decanucleotide of the TR on CAT activity after cotransfection with pRcCMV-TSEP-1, which encodes the rat Y-box protein, in FRT thyroid cells. Mutations of the decanucleotides of the TR are denoted in Figure 39A, as is the sequence of wild type promoter and the location of each decanucleotide and the CRE-like site of the TSHR. Figure 39B shows the raw data of a representative experiment; Figure 39C presents the CAT activity relative to the p8CAT promoter-less control, whose activity is arbitrarily set at one. All cells were cotransfected with pSVGH and conversion rates were normalized to growth hormone (GH) levels. Cell lysates were prepared 48 h after transfection with the TSHR promoter-CAT chimeras indicated plus pRcCMV-TSEP-1 (black bars) or its control, pRc/CMV

(white bars), which was used to construct the TSEP-1 expression vector. Activity in Figure 39C is the mean ± S.E. from four separate experiments. A significant ($p<0.01$) Y-box (TSEP-1)-induced decrease in CAT activity is noted by 2 stars. Mutation of the 5' decanucleotide is Mt-1 or Mt-2, result in a loss of TSHR suppression by TSEP-1.

Figures 40A-A'-40D-D' show the ability of pRcCMV-TSEP-1 to suppress expression, in FRTL-5 or FRT cells, of TSHR promoter-CAT chimeras containing the downstream (S-box) or upstream (TIF-associated) Y-box binding sites. In Figure 40A, the TSHR promoter chimera, pTRCAT5'-220 (Shimura, Y., et al. (1994) J. Biol Chem. 269, 31908-31914) was cotransfected into FRTL-5 cells with either the pRcCMV-TSEP-1 (white bar) or its control plasmid, pRc/CMV. All cells were cotransfected with plasmid pSVGH; cell lysates were prepared 72 h after transfection and conversion rates were normalized to GH levels. In the upper part of the panel, CAT activities are presented relative to that of the p8CAT promoter-less control, whose activity is arbitrarily set at one; in the lower portion, CAT activities are presented as the ratio of activity in the presence of the TSEP-1 vector vs the control vector [TSEP (+)/TSEP(-)]. A significant Y-box (TSEP-1)-induced decrease in CAT activity by comparison to the p8CAT control is noted by a star ($p<0.05$). In Figure 40B and Figure 40C, CAT activities of pTRCAT5'-177, 5'-146, 5'-131, 5'-90 or a p8CAT control are shown as the ratio of activity in cells cotransfected with pRcCMV-TSEP-1 or its pRc/CMV control, [TSEP(+)/TSEP(-)], when cotransfection were performed in FRTL-5 (Fig. 40B) or FRT cells (Fig. 40C). All cells were cotransfected with plasmid pSVGH. In the case of FRT cells, cell lysates were prepared 48 h after transfection, in FRTL-5 cells 72 h after transfection; conversion rates were normalized to

° GH levels. A significant TSEP-1-induced decrease in CAT activity by comparison to the p8CAT

control is noted by a star ($p<0.05$). In Figure 40D, chimeras was created by ligating oligonucleotide TIF containing the TSHR insulin response element sequence , -220 to -188 bp (Shimura, Y., et al. (1994) J. Biol Chem. 269, 31908-31914), to the pCAT-Promoter plasmid from Promega. Constructs are diagrammatically represented by arrows and (+) designations to depict the direction and number of the insulin response element sequences. TIF chimeras or the pCAT control were cotransfected with pRcCMV-TSEP-1 or its pRc/CMV control into FRTL-5 cells and CAT activity analyzed as above. In Figure 40D, bottom, the Y-box (TSEP-1) activity [TSEP-1 (+)] is expressed relative to the activity in the pRc/CMV control transfections [TSEP-1 (-)]. The decrease effected by pRcCMV-TSEP-1 is significant ($P<0.02$). In all experiments denoting relative CAT activity, activities are the mean $\pm$ S.E. from three separate experiments.

Figures 41A-41D depict the downstream silencer of the class I MHC promoter (Figure 41A), its relationship to the interferon response element (Figure 41A), its role and regulation by TSH and/or MMI in relationship to the actions of the transcription factors, TTF-1 and TSEP-1 as modulators of downstream silencer activity (Figures 41B-41D). The downstream silencer involving the CRE is noted in Figure 41A. Its activity as a silencer is lost if the CRE is mutated or deleted as shown in Figure 26A-B. As shown in Figures 26 and 27, EMSA show that TSH and/or MMI treatment of rat FRTL-5 cells increases the formation of a specific DNA complex (arrow) with a 168 bp probe of the class I promoter, -168 to +1 bp, as noted (Figure 41B). Formation of the complex is prevented by including the TSHR insulin response element, oligo TIF (Shimura, Y., et al. (1994) J. Biol Chem. 269, 31908-31914) in the in vitro binding reaction (lane 5) but not by including the thyroglobulin insulin response element, oligo K (Figure 41B; see also Figure 27A). It is not formed using a 168

bp probe which has a nonpalindromic mutation of the CRE, NP CRE, (Figure 41B, lane 6; Figure 27B). Promoter activity of the 127 bp class I promoter-CAT chimera including the downstream silencer is decreased by MMI (Figure 41C). The effect of MMI is lost if oligo TIF is cotransfected with the p(-127)CAT chimera but not if oligo K is cotransfected (Figure 41C, see also Figures 19B and 28B). Cotransfection with a plasmid containing cDNA encoding TTF-1 increases activity, whereas cotransfection with a plasmid with a cDNA encoding TSEP-1 decreases promoter activity (Figure 41C). The silencer interacts with multiple proteins as evidenced by complex formation in Figures 29, 30, 31, and 33, as summarized in Figure 41D; each of these requires an intact CRE to bind to the downstream silencer (Fig. 41D). These have been identified using antibody shift analyses, by direct binding reactions with pure proteins, or by competition with oligonucleotides known to bind them: TTF-1, CREB, Pax-8, SSBP, and TSEP-1 (Y-box protein). Complexes with two of these, TTF-1 and TSEP-1, are increased by TSH or decreased by TSH, respectively (Figure 33). Thus, TSH by decreasing TTF-1 will decrease class I expression by decreasing the enhancer action of TTF-1 (Figure 41C). TSH, by increasing TSEP-1 complex formation will increase its suppressor function (Figure 41D) and decrease class I expression. MMI has effects on each of these transcription factors the same as TSH.

DETAILED DESCRIPTION OF THE INVENTION

For the purpose of a more complete understanding of the invention the following definitions are described herein. Mammal includes, but is not limited to, humans monkeys, dogs, cats, mice, rats, hamsters, cows, pigs, horses, sheep and goats. Drug includes, but is not limited to, MMI, MMI derivatives, CBZ, PTU, thioureylenes, thiones and thionamides. Other candidate drugs include aminothiazole, 1,1,3-tricyano-2-amino-1-propene,

phenazone, thioureas, thiourea derivatives, goitrin derivatives, thiouracil derivatives, sulfonamides, aniline derivatives, derivatives of perchloric acid, iodide, thiocynanates, carbutamide, para-aminobenzoic acid, para-aminosalicylic acid, amphenone B, resorcinol, phloroglucinol, and 2-4-dihydrobenzoic acid, all of which have been noted to have goitrinogen activity and suppress thyroid function. One skilled in the art will also understand that other drugs may be developed by the in vivo and in vitro assays described in examples 2 through 11. These drugs may be natural, synthetic or recombinant in origin. By a drug capable of suppressing expression of MHC Class I molecules we mean a drug that has the capability of decreasing or abolishing MHC Class I cell surface molecules on mammalian cells treated with the drug relative to mammalian cells not treated with the drug. Major histocompatibility complex (MHC) is a generic designation meant to encompass the histocompatibility antigen systems described in different species, including the human leukocyte antigens (HLA). Tissue, includes, but is not limited to, single cells, cells, whole organs and portions thereof. Transplantation rejection includes, but is not limited to, graft versus host disease and host versus graft disease. Autoimmune disease includes, but is not limited to, autoimmune dysfunctions and autoimmune disorders.

By functional equivalents is meant any material which has substantially the same activity as the material to which it is equivalent. By way of example, material may include, but is not limited to, nucleic acid sequences, genes, oligonucleotides, or proteins.

This invention provides a method for treating autoimmune disease and for preventing or treating rejection of a tissue in a transplant recipient. More specifically this invention relates to methods for administering to a mammal in need of such treatment a drug

or drugs capable of suppressing expression of MHC Class I molecules.

Examples of autoimmune diseases that can be treated by this method include, but are not limited to, rheumatoid arthritis, psoriasis, juvenile diabetes, primary idiopathic myxedema, systemic lupus erythematosus, De Quervains thyroiditis, thyroiditis, autoimmune asthma, myasthenia gravis, scleroderma, chronic hepatitis, Addison's.disease, hypogonadism, pernicious anemia, vitiligo, alopecia areata, ectopic dermatitis, Coeliac disease, autoimmune enteropathy syndrome, idiopathic thrombocytic purpura, acquired splenic atrophy, idiopathic diabetes insipidus, infertility due to antispermatazoan antibodies, sudden hearing loss, sensoneural hearing loss, Sjogren's Syndrome, myositis, polymyositis, autoimmune demyelinating diseases such as multiple sclerosis, transverse myelitis, ataxic sclerosis, pemphigus, progressive systemic sclerosis, dermatomyositis, polyarteritis nodosa, chronic hepatitis, hemolytic anemia, progressive systemic sclerosis, glomerular nephritis and idiopathic facial paralysis. Preferred drugs for treating autoimmune diseases by this method are MMI, MMI derivatives, CBZ, and PTU.

In a preferred embodiment, the MHC Class I suppressing drug MMI is administered to a mammal, preferably a human, afflicted with an autoimmune disease. Suitable therapeutic amounts of MMI are in range of about 0.01 mg to about 500 mg per day. A preferred dosage is about 0.1 mg to about 100 mg per day and a more preferable dosage is about 2.5-50 mg per day. Suitable therapeutic amounts of CBZ are in the same range as MMI. The dosage can be administered daily, in approximately equally divided amounts at 8-hour intervals or with breakfast, lunch and dinner. The preferred maintenance dose for adult is 5-15 mg per day for periods of up to one year. Therapy can be continuous, for example about 2.5-30 mg per

day for periods up to one year. Alternatively, therapy can be tapered, for example, 50-100 mg per day at the start, tapering to 5-10 mg per day within 4 to 10 weeks according to thyroid hormone (thyroxin (T_4) or triiodothyronine (T_3)) or thyroid stimulating hormone (TSH) levels in an individual receiving such treatment. Alternatively PTU is administered to a mammal, preferably a human, afflicted with an autoimmune disease. Suitable therapeutic amounts of PTU may be in the range 0.1 mg-2000 mg per day. A preferred dosage for PTU is in a range ten-fold higher than the dosage ranges described above for MMI. The preferred maintenance dose of MMI for children is 0.4 mg per kg, divided into three daily doses at eight hour intervals initially, then half the initial dose to maintain as preferred. It is understood by one skilled in the art that the dosage administered to a mammal afflicted with an autoimmune disease may vary depending on the mammals age, severity of the disease and response to the course of treatment. One skilled in the art will know the clinical parameters to evaluate to determine proper dosage for an afflicted mammal.

In another preferred embodiment, MMI is administered to a mammal, preferably a human, afflicted with systemic lupus erythematosus (SLE). A preferred therapeutic amount is in the range of about 2.5-50 mg per day, administered over 6-12 months, but can be administered in discontinuous treatment periods of similar length over a five year period or for as long as necessary. Alternatively, MMI may be administered in conjunction with the current therapies for SLE, hydrocortisone and cytotoxic drugs, to suppress the disease. SLE patients with breast cancer cannot be readily treated with radiotherapy since they are already immunosuppressed by the ongoing treatment for SLE. Also SLE may be associated with unusual sensitivity to radiation complications therefore radiotherapy exacerbates

the disease. It is anticipated that use of MMI to treat SLE individuals with breast cancer will allow radiotherapy to be administered to such individuals without exacerbation of their condition or the radiation complications.

In another embodiment, a MHC Class I suppressing drug is administered to a mammal, preferably a human, afflicted with an autoimmune disease associated with the development of thyroid autoantibodies in the sera of these animals.

In another embodiment, a MHC Class I suppressing drug is administered to a mammal, preferably a human, afflicted with an autoimmune disease characterized by the development of receptor autoantibodies. For example, autoimmune asthma is associated with ß-adrenergic receptor autoantibodies. Treatment with a MHC Class I suppressing drug, preferably MMI, will alleviate the disease. Another example of such an autoimmune disease is Myasthenia Gravis. Myasthenia Gravis is associated with acetylcholine receptor autoantibodies. Individuals afflicted with myasthenia gravis have a higher frequency of thyroid autoimmunity. Because of the structural and functional relationship between the TSH and acetylcholine receptors, treatment of an animal, preferably a human, afflicted with Myasthenia Gravis with a MHC Class I suppressing drug will help suppress the disease.

The MHC Locus in all mammalian species contains numerous genes and is highly polymorphic. In humans the HLA Complex contains the HLA-A, HLA-B and HLA-C genes which encode Class I HLA molecules and the HLA-DR, HLA-DQ and HLA-DP genes which encode the Class II molecules. Different HLA molecules bind different antigens. Specific HLA antigens have been associated with a predisposition to a particular disease. For example, Ankylosing spondylitis is associated with HLA-B27, rheumatoid arthritis with HLA-DR4 and insulin-dependent diabetes mellitus with HLA-DR3

and, HLA-DR4. ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr, A.I. (eds), Appelton and Lange, Norwalk, Connecticut/San Mateo, California). Although, insulin-dependent diabetes mellitus is negatively associated with HLA-DR2 ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr, A.I. (eds), Appelton and Lange, Norwalk, Connecticut/San Mateo, California) disease expression has been linked to the insulin response element -A binding protein (IRE-ABP) because of its chromosomal base near the HLA complex genes.

Individuals who are HLA-B35 negative, a Class I haplotype, are at low risk of developing scleroderma. Among individuals who develop scleroderma and are HLA-B35 positive, 80% will develop thyroid autoimmune disease and/or will develop thyroid antibodies. In addition, these individuals, are particularly susceptible to human immunodeficiency virus (HIV) infection with rapid disease progression (Kaplan, C et al., (1990) Hum. Hered 40:290-298; Cruse, J.M. et al., (1991) Pathology, 59:324-328; Itescu, S. et al., (1991) J. of Acquired Immune Deficiency Syndrome 5: 37-45; Scorza, R. et al., (1988) Human Immunology 22:73-79). MHC Class I suppressing drugs should mitigate the symptoms not only of scleroderma but also mitigate progression of HIV, allowing for a better prognosis for these individuals. In a preferred embodiment the MHC Class I suppressing drug used to treat individuals afflicted with HIV is MMI. A preferred therapeutic amount is in the range of about 5-50 mg per day.

In another embodiment, MMI is administered to a mammal, preferably a human, afflicted with an autoimmune disease as an adjunct therapy in the treatment of an autoimmune disease. For example, De Quervains thyroiditis is currently treated with hydrocortisone or salicylates; it is anticipated that the addition of MMI plus

hydrocortisone or salicylates will more efficiently suppress the disease.

In another embodiment, MMI and thyroid hormone are co-administered to a mammal in need of such treatment so as to compensate for suppression of thyroid hormone production by MMI. The thyroid hormones thyroxin (T_4) or triiodothyronine (T_3) may be co-administered with MMI; thyroxin co-administered with MMI is preferable. A preferred dose of thyroxin is about 0.01 to 0.5 mg per day and a more preferable dosage is about 0.1 to 0.3 mg per day.

The method of this invention is also suitable for preventing or treating rejection of a transplanted tissue in a recipient mammal, preferably a human. Examples of tissues which may be transplanted include, but are not limited to, heart, lung, kidney, bone marrow, skin, pancreatic islet cells, thyroid, liver and all endocrine tissues, neural tissue, muscle, fibroblast, adipocytes, and hermatopoetic stem cells.

In a preferred embodiment, pancreatic islet cells are isolated from a donor and treated with MMI prior to transplantation into a recipient suffering from diabetes. Diabetes is caused by loss of islet cells as a result of autoimmune disease. Transplantation of islet cells will correct such a deficiency. Islet cells may be treated with about 0.1 to about 50 mM MMI. The islet cells are preferably treated with about 0.1 to about 10 mm MMI, in the form of an aqueous solution for 24 to 72 hours or longer as necessary to suppress expression of MHC Class I molecules on the islet cells. After transplantation the recipient may be further treated with MMI or MMI and hydrocortisone or MMI and immunosuppressive agents.

Tumors of certain tissues can be treated by total destruction of that tissue and associated tumor. For example, thyroid tumors are treated with radioiodine to destroy both normal and diseased tissue to stop the

progression of the disease. Continuous cultures of normal human thyroid cells are now available. In another embodiment of this invention, these human cells could be treated with MMI to suppress MHC Class I expression and transplanted into a recipient in need of thyroid cells. This technology would provide human donor cells for transplantation on demand since cells could be maintained in culture, treated with MMI and transplanted into a recipient.. In yet another embodiment, the nucleic acid sequences for a Sox-4 protein or the functional equivalent thereof, or a Y-box protein or the function equivalent thereof may be introduced into human cells by conventional methodology including, but not limited to, micro injection, electroporation, viral transduction, lipofection, calcium phophate, particle mediated gene bombardment, gene transfer or direct injection of nucleic acid sequences encoding the Sox-4 or a Y-box protein or functional equivalents thereof, or any other procedures known to one skilled in the art. Examples of vectors that can be used to express the Sox-4 or a Y-box protein or functional equivalents thereof include, but is not limited to, retroviral vectors, herpes virus vectors, fowlpox virus vectors, adeno associates virus vectors (AAV) or plasmids. Such vectors may have tissue specific promoters or ubiquitous promoters known to those skilled in the art. The mammalian cells expressing the Sox-4 or Y-box proteins or the functional equivalents thereof will have suppresed expression of Class 1 molecules thereby preventing or inhibiting transplantation rejection. Further, these human cells may be from noncogeneic individuals, since suppression of MHC Class I by MMI will reduce the possibility that the immune system of the recipient will recognize these cells as "nonself".

In another embodiment of this invention, whole organs may be pretreated by perfusion with MMI to suppress MHC Class I expression. As a drug of low molecular weight

MMI will readily perfuse the organ, cross blood vessel barriers and thereby act on most or all cells in the organ. This would reduce or avoid the need for exact matches of donor and recipient in transplantation.

In another embodiment, post transplantation individuals are treated with MMI and hydrocortisone. Hydrocortisone or hydrocortisone in conjunction with other immunosuppressive agents is currently used as a therapy for individuals after transplantation. Hydrocortisone and other hormones are additive with MMI in their effect on MHC Class I levels. Thus it is anticipated pretreatment with MMI, plus treatment with MMI and hydrocortisone or MMI, hydrocortisone and other immunosuppressive agents after transplantation will reinforce self tolerance.

In another embodiment of this invention, MMI is used to pretreat cells containing a recombinant gene, so that the cells may be transplanted into a mammal, preferably a human in need of gene therapy. To provide gene therapy to an individual, a genetic sequence which encodes a desired protein is inserted into a vector and introduced into a host cell. Examples of diseases that may be suitable for gene therapy include, but are not limited to sickle cell anemia, cystic fibrosis, ß-thalassemia, hemophilia A and B, glycosyl transferase enzyme defects, and cancer. Examples of vectors that may be used in gene therapy include, but are not limited to, defective retroviral, adenoviral, or other viral vectors (Mulligan, R.C. (1993) Science Vol. 260: 926-932). The means by which the vector carrying the gene may be introduced into the cell include, but is not limited to, electroporation, transduction, or transfection using DEAE-dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art (Sambrook, J. et al. (1989) in "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Press, Plainview, New York).

Examples of cells into which the vector carrying the gene may be introduced include, but are not limited to, continuous cultures of normal human cells, such as human pancreatic islet or thyroid cells or continuous cultures of normal mammalian cells such as rat FRTL-5 cells. In a preferred embodiment, FRTL-5 rat thyroid cells containing a recombinant gene under a thyroid specific promoter are treated with MMI to suppress MHC Class I. The treated cells are transplanted into a mammal, preferably a human, and secrete factors able to control the disease. Such cells can be maintained in prolonged culture, in a functioning growing state and treated with MMI or MMI with hormone supplementation to suppress Class I. Cells carrying a variety of recombinant genes could be readily available on demand. Elimination of the need for autologous cells would allow a major advance in transplantation.

In another embodiment of this invention an in vivo assay is used to assess the ability of a candidate drug to suppress MHC Class I expression. In the first step of the assay, the role of MHC Class I in a particular autoimmune disease is evaluated by determining if the symptoms or signs of that particular autoimmune disease can be induced in MHC Class I-deficient mice. Lack of inducibility of the autoimmune disease in MHC Class I-deficient mice would suggest a role for MHC Class I in that disease. Examples of MHC Class I-deficient mice which can be used include, but are not limited to, MHC Class I-deficient mice generated by homologous recombination, MHC Class I-deficient mice created by insertion of transgenes, and MHC Class I-deficient mice created by chromosomal loss. MHC Class I-deficient mice are also commercially available. Methods by which the autoimmune disease can be recreated in these mice include, but are not limited to, viral infection, induction by antibodies and induction by chemicals or other

environmental agents. Alternatively, Class I-deficient animals can be mated with spontaneous autoimmune animal models and the resulting progeny analyzed for autoimmune disease.

In a preferred embodiment MHC Class I deficient mice are immunized with a human monoclonal anti-DNA antibody bearing a major idiotype, designated 16/6Id. (Shoenfeld, Y. et al. (1983) J. Exp. Med. 158:718-730). In the next step of this embodiment an animal model of the autoimmune disease is exposed to the MHC Class I suppressing drug. Examples of autoimmune animal models include, but are not limited to, transgenic animals, animals generated by homologous recombination, chromosomal loss and animals with naturally or spontaneously occurring disease.

In a preferred embodiment, SLE is experimentally induced in mice. Examples of how SLE is experimentally induced in mice include, but are not limited to, immunization with a monoclonal 16/6 idiotype (Shoenfeld, Y. et al., (1983)), a monoclonal anti-16/6Id antibody (Mendlovic, S. et al. (1989) Eur. J. Immun., 19:729-734) and T cell lines specific for the 16/6 idiotype (Fricke, H. et al., (1991) Immunology, 73:421-427). The strains of mice that may be used include, but are not limited to, Balb, 129, C3H.SW, SJL, AKR, and C3HSW. A preferred method is immunization of mice with a human anti-DNA monoclonal antibody, the 16/6Id antibody (Shoenfeld, Y. et al (1983)). The immunized animals are then exposed to a drug, preferably a MMI analog, and evaluated for alleviation of symptoms of the disease. Parameters evaluated in 16/6Id-treated mice include, but are not limited to, leukopenia, proteinuria, levels of cell surface markers on the peripheral blood lymphocytes (PBL), and immune complex deposits in kidney. Examples of methods for evaluating these parameters include, but are not limited to, analyses of blood cells and sera, tissue

biopsies or extracts, urine analyses and analysis of antibody production and immune activated cells. It will be understood by those skilled in the art that conventional methods can be used to evaluate these parameters. Examples of conventional methods that can be used evaluate these parameters include, but are not limited to, cell counts, ELISAs (Heineman, W.R. et al (1987), Methods of Biochemical Analyses 32:345-393), quantitative protein assays (Ausubel, J. et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York), immunohistology ("Basic and Clinical Immunology" (1991) Stites, A.P. and Terr, A.I. (eds.) Appelton and Lange, Norwalk, Connecticut San Mateo, California), and analysis of cell surface markers on lymphocytes ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr, A.I. (eds), Appelton and Lange, Norwalk, Connecticut/San Mateo, California).

In another embodiment of this invention, another in vivo assay is used to assess and develop drugs capable of suppressing expression of MHC Class I molecules. In this in vivo method a tissue to be transplanted into an animal is pretreated with a MHC Class I suppressing drug. Examples of tissues which can be transplanted include, but are not limited to, thyrocytes, hepatocytes, neural tissue, muscle, fibroblasts, adipocytes, and islet cells, endocrine cells and tissues, thyroid, liver, skin, bone marrow, kidney, lung and heart. In a preferred embodiment rat thyroid FRTL-5 cells are pretreated with a MHC Class I suppressing drug prior to transplantation in a rat or mouse. Examples of the means by which the tissue may be transplanted include, but is not limited to, general surgical procedures, intravenous and subcutaneous injection. In a preferred embodiment rat thyroid FRTL-5 cells are subcutaneously injected into the lower back of a rat or mouse. The pretreated transplanted tissue remains in the recipient animal for periods between 30 - 100 days.

Preferably the state of the transplanted tissue is evaluated 60 days after transplantation. One skilled in the art will understand the conventional methods available to evaluate the transplanted tissue. In a preferred embodiment the site of injection of the pretreated transplanted FRTL-5 cells is excised from the recipient animal. The excised tissue is evaluated microscopically for the presence of FRTL-5 cells. In addition FRTL-5 cells are evaluated for the ability of TSH to cause an increase in cAMP levels and an increase in iodide uptake which are indicative of normal FRTL-5 function. The presence of FRTL-5 cells, that had been treated with the candidate drug prior to transplantation, in the excised tissue and that exhibit the increase in TSH mediated cAMP levels or iodine uptake is predictive of the candidate drug's usefulness for preventing or treating transplantation rejection.

In another embodiment of this invention, in vitro assays are used to assess and develop candidate drugs capable of suppressing expression of MHC Class I molecules. One in vitro assay in the present invention relates to a method for assessing the ability of a candidate drug to suppress expression of MHC Class I molecules by detecting altered binding of a protein or proteins in a mammalian cell extract, from cells treated or not treated with the candidate drug, to a MHC Class I regulatory nucleic acid sequence or the functional equivalent thereof. Extracts from mammalian cells treated with a candidate drug are combined with MHC Class I nucleic acid regulatory sequences and the existence of complexes between said sequences and proteins or protein from the extract is detected. Alterations in binding of mammalian cell protein or proteins to said nucleic acid sequences may be assessed by comparison to binding of protein or proteins to the same MHC Class I regulatory nucleic acid sequence in extracts from untreated cells.

Regulatory nucleic acid sequences are intended to encompass sequences that regulate transcription of a MHC Class I gene or the functional equivalents thereof. By alteration we mean an enhancement or appearance of the signal of the detected complex in treated versus untreated extracts or a decrease or absence of signal of the detected complex in treated versus untreated extracts. Protein extracts may be either nuclear or cellular extracts; cellular extracts are preferable. Cellular or nuclear protein extracts from mammalian cells are generated by conventional methods (Ausuebel, J. et al. (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York).

Examples of nucleic acid sequences that can be used in this in vitro assay include, but is not limited to, nucleic acid fragments containing regulatory sequences of MHC Class I promoters or the functional equivalents thereof. By way of example such fragments may include single or double stranded oligonucleotides. Sequences encoding the regulatory regions of the PD1 silencer elements such as the upstream and downstream silencers may be used in this method. In the PD1 promoter the upstream silencer is located at about -724 to about -697 base pairs and the downstream silencer at about -127 to about -90 base pairs 5′ of the PD1 start site. Also intended to be encompassed by this invention are nucleic acid sequences which are functionally equivalent to the two silencer sequences of the PD1 promoter. In a preferred embodiment the downstream silencer sequences centered on the CRE at -107 to -100bp or their functional equivalents are used in the in vitro assays described herein.

Examples of additional nucleic and sequences that may be used in the in vitro assay include, but is not limited to regulatory sequences of the MHC Class I promoter encoding for enhancer regions. By way of example sequences including the upstream and downstreams enhancers

of the PD1 MHC Class I promoter, or their functional equivalents may be used in the in vitro assays of this invention. In the PD1 promoter the upstream enhancer overlaps with the upstream silencer (See Figure 9); the downstream enhancer is 5' to the interferon response element (Figure 16B). Examples of proteins that may form complexes with the upstream silencer and enhancer include, but is not limited to, Sox-4, C-jun family members, c-fos family members, NF-κB and its subunits or the functional equivalents thereof. Examples of proteins that may form complexes with the downstream enhancer (enhancer A) include, but are not limited to, Sox 4, NFκ-B and its subunits, c-fos family members, Pax 8, a TTF-1 protein, a Y-box protein, such as TSEP-1, or the functional equivalents thereof. Candidate drugs capable of suppressing MHC Class 1 molecules should decrease or abolish complex formation with the upstream or downstream enhancer sequences.

Examples of mammalian cells that can be used in this in vitro assay include, but are not limited to, mammalian cell thyrocytes, hepatocytes, neural tissue, muscle, fibroblasts, adipocytes, and HELA cells. Rat FRTL-5 thyroid cells are preferable (American Type Culture Collection, Rockville, Maryland, ATCC-CRL 8305).

In a one embodiment, the nucleic acid sequences used in this assay are derived from sequences homologous to the DNA regulatory sequences of the MHC Class I gene, PD1. In a preferred embodiment, these nucleic acid sequences are DNA fragments 114 (bases 221 to 320 of SEQ ID NO:1), 140 (bases 321 to 455 of SEQ ID NO:1) and 238 (bases 456 to 692 of SEQ ID NO:1), as shown in Figure 9. The double-stranded oligonucleotides shown in Figure 10 and designated S1, S2, S3, S5-8 (SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:10, SEQ ID NO:5-SEQ ID NO:8) may also be used or the double-stranded oligonucleotide(K) (SEQ ID NO:38). The K oligonucleotide (SEQ ID NO:38) is the TTF-2/Sox-4

reactive element or regulatory nucleic acid sequence which is in the thyroglobulin promoter (Santisteban, P. et al (1992) Mol. Endocrinol:6:1310-1317) and which is related in sequence to the Sox-4 like binding site in the silencer complex (Figure 10).

In a preferred embodiment the ability of a drug to suppress expression of MHC Class I molecules is measured by decreased or increased binding of a protein or proteins in the extract to the above described MHC Class I regulatory nucleic acid sequences or to single or double stranded oligonucleotides or their functional equivalents. By decreased binding we mean a diminution or loss of signal or absence of signal of the detected complexes in treated versus untreated extracts. By increased binding we mean the appearance of or an increase of signal of the complexes in treated versus untreated cells. By complex we mean protein or proteins bound to the nucleic acid sequence.

The protein or proteins which form the complex with the nucleic acid sequences may be ubiquitously expressed or tissue specific. Such proteins may directly bind to the nucleic acid sequences or interact or complex with proteins capable of binding this nucleic acid sequences. Intended to be encompassed by this definition are proteins capable of binding single or double stranded nucleic acid sequences. By way of example the proteins forming the complex in cells with the upstream silencer-enhancer of a MHC Class I gene may comprise, but is not limited to, the NF-KB and its subunits (p65/p50 subunits), c-fos related proteins or family members, C-jun related proteins or family members, a Sox-4 protein or the functional equivalents thereof which possess the substantially equivalent biological activity of such proteins. (Example 8; Kieran, M., et al., (1990) Cell 62, 1007-1018; Ghosh, S., et al., (1990) Cell 62, 1019-1029; Ryseck, R.-P., et al., (1992) Mol. Cell. Biol. 12, 674-

684; Stein, B., Ert al., (1993) EMBO J. 12, 879-3891; Stein, B., et al., (1993) Mol. Cell. Biol. 13, 964-3974; Nishina, H., et al., (1990) Proc. Natl. Acad. Sci. U. S. A. **87,** 3619-3623; Baldwin, A. et al., (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 723-727; Fujita, T., et al., (1992) Genes and Develop. 6, 775-787; Giuliani, C., et al. (1995) J. Biol. Chem. 270, 11453-11462). One of skill in the art will appreciate that a variety of factors such as cell or tissue type will determine the exact make up of the proteins interacting or forming the complex. By way of example the proteins forming a complex with the downstream silencer in cells may comprise, but is not limited to, a thyroid transcription factor -1, (TTF-1), Pax 8, a Y-box protein, a single stranded binding protein (SSBP) and a cyclic AMP regulatory binding protein (CREB) or the functional equivalents thereof which posses substantially equivalent biological activity of such proteins. (See Example 9, 11); Civitareale, D., et al., (1993) Mol. Endocrinol. 7, 1589-1595; Civitareale, D., et al., (1989) EMBO J. , 2537-2542; Guazzi, S., et al., (1990) EMBO J. 9, 631-3639; Francis-Lang, H., et al., (1992) Mol. Cell. Biol. 12, 576-588; Zannini, M., et al., (1992) Mol. Cell. Biol. 12, 4230-4241; Shimura, H., et al., (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al., (1995) Endocrinology, 136, 269-282) Davis, T. L., et al., (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9682-9686; Kinniburgh, A. J. (1989) Nucleic Acids Res. 17, 7771-7778; Postel, E. H., et al., (1989) Mol. Cell. Biol. 9, 5123-5133; Kolluri, R., et al., (1992) Nucleic Acids Res. 20, 111-116; Johnson, A. C., et al., (1988) Mol. Cell. Biol. 8, 4174-4184; Pestov, D. G., et al., (1991) Nucleic Acids Res. 19, 6527-6532; Hoffman, E. K., et al., (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2705-2709; Wolffe, A. P., et al., (1992) New Biol. 4, 290-298; Kolluri, R., et al., (1991) Nucleic Acids Res. 19, 4771; Ozer, J., et al., (1990) J. Biol. Chem. 265, 22143-22152; Faber, M., et

al., (1990) J. Biol. Chem. 265, 22243-22254; Petty, K. J., et al., GenBank Accession Number M69138; Didier, D. K., et al., (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7322-7326; Sakura, H., et al., (1988) Gene 73, 499-507; Spitkovsky, D. D., et al., (1992) Nucleic Acids Res. 20, 797-803; Sabath, D. E., et al., (1990) J. Biol. Chem. 265, 12671-12678; Giuliani, C., et al., (1995) J. Biol. Chem. 270, 11453-11462) Montminy, M. R., et al., (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6682-6686; Angel, P., et al., (1987) Mol. Cell. Biol. 7; 2256-2266; Leonard, J., et al., (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6247-6251; Vallejo, M., et al., (1992) J .Biol. Chem. 267; 12868-12875; Leonard, J., et al., (1993) Mol. Endocrinol. 7, 1275-1283; Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 1701-1715; Habener, J. F. (1990) Mol. Endocrinol. 4, 1087-1094).

Detection of the complexes can be carried out by a variety of techniques known to one skilled in the art. Detection of the complexes by signal amplification can be achieved by several conventional labelling techniques including radiolabels and enzymes (Sambrook, T. et al (1989) in "Molecular Cloning, A Laboratory Manual," Cold Spring Harbor Press, Plainview, New York). Radiolabelling kits are also commercially available. Preferred methods of labelling the DNA sequences are with ^{32}P using Klenow enzyme or polynucleotide kinase. In addition, there are known non-radioactive techniques for signal amplification including methods for attaching chemical moieties to pyrimidine and purine rings (Dale, R.N.K. et al. (1973) Proc. Natl. Acad. Sci., 70:2238-2242; Heck, R.F. (1968) S. Am. Chem. Soc., 90:5518-5523), methods which allow detection by chemiluminescence (Barton, S.K. et al. (1992) J. Am. Chem. Soc., 114:8736-8740) and methods utilizing biotinylated nucleic acid probes (Johnson, T.K. et al. (1983) Anal. Biochem., 133:126-131; Erickson, P.F. et al. (1982) J. of Immunology Methods, 51:241-249; Matthaei,

F.S. et al. (1986) Anal. Biochem., 157:123-128) and methods which allow detection by fluorescence using commercially available products. Non-radioactive labelling kits are also commercially available. Methods useful to detect complexes of protein extract bound to DNA fragments or double-stranded oligonucleotides include mobility-shift analysis, Southwestern, and immunoprecipitation (Sambrook, J. et al., (1989); Ausubel, J. et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York). A preferred method is gel mobility-shift analysis using a radiolabelled double-stranded nucleic acid sequence. For mobility shift analysis, the protein extract-oligomer complexes can also be detected by using labelled protein extract, wherein the cells can be metabolically labelled with ^{35}S, or tritiated thymidine. Alternatively, radioiodination with ^{125}I or non-radioactive labelling using biotin and various fluorescent labels prior to the preparation of the protein extract may also be used.

Another in vitro assay of the invention relates to a method for assessing the ability of a drug to suppress expression of MHC Class I by measuring the activity of a reporter gene operably linked downstream of a MHC Class I promoter and its regulatory sequences or the functional equivalents thereof. The reporter gene operably linked to a MHC Class I promoter and its regulatory sequence is introduced into mammalian cells, said mammalian cells are treated with the candidate drug and the activity of the reporter gene in lysates from treated and untreated mammalian cells is measured. A decrease of activity of the reporter gene in cell lysates from treated versus nontreated cells is predictive of the usefulness of the candidate drug in suppressing MHC Class I expression.

Preferred regulatory sequences that may be operably linked to the reporter gene are sequences

corresponding to the silencer and enhancer regions of the MHC Class I, PD1 gene. By way of example, these sequences may include, but are not limited to, the 114 (bases 221 to 230 of SEQ ID NO:1), 140 (bases 321 to 455 of SEQ ID NO:1), 151 (bases 54 to 220 of SEQ ID NO:1) and 238 (bases 456 to 692 of SEQ ID NO:1) sequences or the functional equivalents thereof, as shown in Figures 9A-9B, with their cognate promoters. In addition sequences corresponding to the downstream silencer region -127 to -90 bp or -127 to -80 bp or the functional equivalents thereof may also be used. It will be understood by one skilled in the art that sequentially and functionally homologous regions found in the regulatory and promoter domains of other Class I genes may also be used and are intended to be encompassed by the invention. Examples of reporter genes include, but are not limited to, the chloramphenicol acetyltransferase (CAT) gene, the β-galactosidase gene, the luciferase gene and human growth hormone (hGH) (Sambrook, J. et al. (1989); Ausubel, F. et al. (1987) in "Current Protocols in Molecular Biology" Supplement 14, section 9.6 (1990); John Wiley and Sons, New York). Examples of mammalian cells that can be used in this in vitro assay include, but are not limited to, mammalian cell thyrocytes, hepatocytes, neural tissue, muscle, fibroblasts, adipocytes, and HELA cells. The means by which the regulatory sequence operably linked to the reporter gene may be introduced into cells are the same as those described above. In a preferred embodiment the CAT gene is operably linked to one of the above mentioned PDI sequences and introduced into FRTL-5 cells.

It is understood by one skilled in the art that the ability of a candidate drug to suppress expression of MHC Class I molecules can also be assessed by comparing levels of cellular mRNA in mammalians cells treated with the candidate drug versus cells not treated with the candidate drug. Examples of methods for determining

cellular mRNA levels include, but is not limited to Northern blotting (Alwine, J.C. et al. (1977) Proc. Natl. Acad. Sci., 74:5350-5354), dot and slot hybridization (Kafatos, F.C. et al. (1979) Nucleic Acids Res., 7:1541-1522), filter hybridization (Hollander, M.C. et al. (1990) Biotechniques; 9:174-179), RNase protection (Sambrook, J. et al. (1989) in "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Press, Plainview, NY), polymerase chain reaction (Watson, J.D. et al.) (1992) in "Recombinant DNA" Second Edition, W.H. Freeman and Company, New York) and nuclear run-off assays (Ausubel, F. et al. (1989) in "Current Protocols in Molecular Biology" Supplement 9 (1990); John Wiley and Sons, New York). Conventional methodology known to those skilled in the art can be used to assess the mRNA levels or rate of transcription of a given gene (Sambrook, J. et al. (1989) in "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Press, Plainview, NY).

In yet another in vitro assay the ability of a candidate drug to suppress MHC Class I expression is evaluated by assessing a drug's ability to alter the expression of one or more of the proteins capable of modulating MHC Class I expression or their corresponding RNA. By way of example such proteins may include, but are not limited to, a Sox-4 protein or the functional equivalent thereof, TTF-1 thyroid transcription factor or the functional equivalent thereof, a single stranded binding protein (SSBP) such as SSBP or the functional equivalent thereof, and a Y-box protein or the functional equivalent thereof. In a preferred embodiment the levels of expression of the mRNA for the Sox-4 protein and a Y-box protein, designated TSEP-1 described herein are assessed in cells exposed to the candidate drug. Preferably rat FRTL-5 cells are used. Conventional methodology may be used to assess the rate of transcription of these genes or the levels of the mRNA for

these genes present in a cell (Ausubel, F. et al. (1989) in "Current Protocols in Molecular Biology" (1987); John Wiley and Sons, New York). Examples of such methods include, but are not limited to, Northern Blot Analysis, or Polymerase Chain Reaction (PCR). A drug capable of suppressing MHC Class I expression should also suppress or decrease SSBP messenger RNA (mRNA), Sox-4 mRNA, or TTF-1 mRNA levels. Alternatively the level of the Sox 4 or TTF-1 protein may be evaluated as an indicator of the therapeutic potential of a candidate drug. A drug capable of suppressing MHC Class I molecules should decrease the levels of SSBP or TTF-1 protein. Evaluation of protein levels may be assessed by conventional methodology known to those skilled in the art including, but not limited to, Western Blot Analysis, ELISA (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, New York; Sambrook et al. (1989) in "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Press, Plainview, New York). Alternatively, the levels of expression of the RNA or proteins of a Y-box protein or the functional equivalent thereof may be evaluated for the therapeutic potential for candidate drug. The RNA or protein levels of Y-box protein should increase if a drug is capable of suppressing MHC Class I molecules. Conventional methodology known to those skilled in the art or described herein may be used in this assay.

In yet another embodiment the therapeutic potential of a candidate drug ability to MHC Class I may be evaluated by the ability of the drug to evaluate the oxidation/reduction state of proteins capable of modulating Class I expression. By way of example such protein may be SSBP, a TTF-1 protein, Y-Box proteins such as TSEP-1, a Pax8 protein, a CREB protein, NF-κB and its subunits, fos family members or the functional equivalents thereof. By way of example the effect of the drug on

enzymes within a cell capable of modulating or altering the oxidation/reduction state of a protein capable of also modulating MHC Class I expression may be assessed. Alternatively, the activity of the enzymes responsible for modulating the oxidation/reduction state of the proteins capable of modulating MHC Class I can be assessed. Examples of such enzymes include, but are not limited to, thioredoxin, superoxide dismutase or the functional equivalents thereof. By way of example, assays that may be used in this method included, but not limited to, the assays described in Noiva, R. (1994) Protein Expr. Purif. 5, 1-13; Tonissen, K. et al., (1993) J. Biol. Chem. 268, 22485-22489; Hayashi, T., et al., (1993) J. Biol. Chem. 268, 11380-11388; Okamato, T., et al., (1992) Int. Immunol. 4, 811-819, herein incorporated by reference.

The present invention also provides nucleic acid sequences which encode proteins capable of modulating MHC Class I expression. In particular, this invention provides nucleic and amino acid sequences for a Sox-4 protein (Example 8) and a Y-box protein designated TSEP-1 (Example 11).

The nucleic acid sequence for the Sox-4 protein shown in Figure 20, and the nucleic acid sequence for the Y-Box protein, designated TSEP-1, shown in Figure 38 represent preferred embodiments of the invention. It is, however, understood by one skilled in the art that due to the degeneracy of the genetic code variations in the cDNA sequence shown in Figures 20 and 38 will still result in a DNA sequence capable of encoding the Sox-4 or TSEP-1 respectively protein. Such DNA sequences are therefore functionally equivalent to the sequence set forth in Figure 20 and 28 and are intended to be encompassed within the present invention. Further, a person of skill in the art will understand that there are naturally occurring allelic variations in a given species of the nucleic acid sequences shown in Figures 20 and 28, and that these

variations are also intended to be encompassed by the present invention.

The predicted Sox-4 protein is about 53 kilodaltons and the predicted TSEP-1 protein is about 42 kilodaltons (kd). This invention further includes protein or peptides or analogs thereof having substantially the same function as the Sox-4 or TSEP-1 proteins. Such proteins or polypeptides include, but are not limited to, a fragment of the protein, or a substitution, addition or deletion mutant of the Sox-4 or TSEP-1 protein. This invention also encompasses proteins or peptides that are substantially homologous to these proteins.

The term "analog" includes any polypeptide having an amino acid residue sequence substantially identical to the Sox-4 or TSEP-1 sequences specifically shown herein Figures 20 and 38 in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the functional aspects of the Sox-4 or TSEP-1 protein antigen as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue, such as isoleucine, valine, leucine, or methionine, for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

Intended to be included in this invention, are conservatively substituted variations of the Sox-4 and TSEP-1 proteins described herein. The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue. "Chemical derivative" refers to a subject polypeptide having one or more residues chemically

derivatized by reaction of a functional side group. Examples of such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those proteins or peptides which contain one or more naturally-occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Proteins or polypeptides of the present invention also include any polypeptide having one or more additions and/or deletions of residues relative to the sequence of a polypeptide whose sequence is encoded in the DNA for the Sox-4 or TSEP-1 protein, so long as the requisite activity is maintained.

This invention also provides a recombinant DNA molecule comprising all or part of the Sox-4 nucleic acid sequence and a vector or all or part of the TSEP-1 nucleic acid sequence and a vector. Expression vectors suitable for use in the present invention comprise at least one expression control element operationally linked to the nucleic acid sequence. The expression control elements are inserted in the vector to control and regulate the expression of the nucleic acid sequence. Examples of expression control elements include, but are not limited to, lac system, operator and promoter regions of phage lambda, yeast promoters, and promoters derived from

polyoma, adenovirus, retrovirus or SV40. Additional preferred or required operational elements include, but are not limited to, leader sequence, termination codons, polyadenylation signals and any other sequences necessary or preferred for the appropriate transcription and subsequent translation of the nucleic acid sequence in the host system. It will be understood by one skilled in the art the correct combination of required or preferred expression control elements will depend on the host system chosen. It will further be understood that the expression vector should contain additional elements necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the host system. Examples of such elements include, but are not limited to, origins of replication and selectable markers. It will further be understood by one skilled in the art that such vectors are easily constructed using conventional methods (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, New York) or commercially available.

Another aspect of this invention relates to a host organism into which recombinant expression vector containing all or part of the Sox-4 nucleic acid sequence or TSEP-1 nucleic acid sequence or combination thereof, has been inserted. The host cells transformed with the expression vectors of this invention include eukaryotes, such as animal, plant, insect and yeast cells and prokaryotes, such as E. coli. The means by which the vector carrying the gene may be introduced into the cell include, but are not limited to, microinjection, electroporation, transduction, or transfection using DEAE-dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art (Sambrook et al. (1989) in "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Press, Plainview, New York).

In a preferred embodiment, eukaryotic expression vectors that function in eukaryotic cells are used. Examples of such vectors include, but are not limited to, retroviral vectors, vaccinia virus vectors, adenovirus vectors, herpes virus vector, fowl pox virus vector, bacterial expression vectors, plasmids, such as pcDNA3 (Invitrogen, San Diego, CA) or the baculovirus transfer vectors. Preferred eukaryotic cell lines include, but are not limited to, thyroid cells such as FRTL-5 or FRT cells, COS cells, CHO cells, HeLa cells, NIH/3T3 cells, or BRL cells. In a particularly preferred embodiment the recombinant expression vector is introduced into mammalian cells, such as FRTL-5 NIH/3T3, COS, or CHO, to ensure proper processing and modification of the recombinant proteins.

In one embodiment the expressed recombinant TSEP-1 or Sox-4 proteins may be detected by methods known in the art which include Coomassie blue staining and Western blotting using antibodies specific for the TSEP-1 or Sox-4 proteins.

In a further embodiment, the recombinant protein expressed by the host cells can be obtained as a crude lysate or can be purified by standard protein purification procedures known in the art which may include differential precipitation, molecular sieve chromatography, ion-exchange chromatography, isoelectric focusing, gel electrophoresis, affinity, and immunoaffinity chromatography and the like. (Ausubel et. al., (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, New York). In the case of immunoaffinity chromatography, the recombinant protein may be purified by passage through a column containing a resin which has bound thereto antibodies specific for the Sox-4 or TSEP-1 proteins (Ausubel et. al., (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, New York).

The nucleic acid sequences or portions thereof, of this invention are useful as probes for the detection of expression of the Sox-4 or TSEP-1 gene in biological samples. Examples of samples include, but are not limited to, tissues cells, homogenates, extracts, biopsies, fine needle aspirates or tissue slices. Therefore, another aspect of the present invention relates to a bioassay for detecting messenger RNA encoding either the Sox-4 or TSEP-1 proteins in a biological sample comprising the steps of contacting all or part of the nucleic acid sequence of this invention with said biological sample under conditions allowing a complex to form between said nucleic acid sequence and said messenger RNA, detecting said complexes and, determining the level of said messenger RNA. RNA can be isolated as whole cell RNA or as poly(A)$^+$ RNA by conventional methodology.

In another embodiment, combinations of oligonucleotide pairs based on the Sox-4 or TSEP-1 sequence in Figures 20 and 38 are used in a Polymerase Chain Reaction (PCR) as primers to detect Sox-4 or TSEP-1 mRNA respectively. These primers can also be used in a method following the reverse transcriptase - Polymerase Chain Reaction (RT-PCR) process for amplifying selected RNA nucleic acid sequences as detailed in Ausubel et al., (eds) (1987) In "Current Protocols in Molecular Biology" Chapter 15, John Wiley and Sons, New York, New York. The oligonucleotides can be synthesized by automated instruments sold by a variety of manufacturers or can be commercia commercially prepared based upon the nucleic acid sequence of this invention. One skilled in the art will know how to select PCR primers based on the Sox-4 or TSEP-1 nucleic acid sequence for amplifying Sox-4 or TSEP-1 respectively RNA in a sample.

In yet another embodiment of this invention all or parts thereof of the Sox-4 or TSEP-1 nucleic acid sequence can be used to generate transgenic animals.

Preferably the Sox-4 or TSEP-1 gene is introduced into an animal or an ancestor of the animal at an embryonic stage, preferably at the one cell stage and generally not later than about the eight cell stage. There are several means by which transgenic animals carrying a Sox-4 or TSEP-1 gene can be made. One method involves the use of retroviruses carrying all or part of the cell sequence. The retroviruses containing the transgene are introduced into the embryonic animal by transfection. Another method involves directly injecting the transgene into the embryo. Yet another method employs the embryonic stem cell method or homologous recombination method known to workers in the field. Examples of animals into which the transgene can be introduced include, but is not limited to, primates, mice, rats or other rodents. Such transgenic animals may be useful as biological models for the study of autoimmunity, transplantation rejection or cancer and to evaluate diagnostic or therapeutic methods for autoimmunity, cancer or transplantation rejection.

This invention further comprises an antibody or antibodies reactive with either the Sox-4 or TSEP-1 the protein or peptides having the amino acid sequence defined in Figures 20 and 38 or a unique portion thereof. In this embodiment of the invention the antibodies are monoclonal or polyclonal in origin. Sox-4 or TSEP-1 protein or peptides used to generate the antibodies may be from natural or recombinant sources or generated by chemical synthesis. Natural Sox-4 or TSEP-1 proteins can be isolated from mammalian biological samples such as rat thyroid. The natural proteins may be isolated by the same methods described above for recombinant proteins. Recombinant Sox-4 or TSEP-1 proteins or peptides may be produced and purified by conventional methods. Synthetic Sox-4 or TSEP-1 peptides may be custom ordered or commercially made based on the predicted amino acid sequences of the respective proteins provided the present

invention or synthesized by methods known to one skilled in the art (Merrifield, R.B. (1963) J. Amer. Soc. 85:2149). If the peptide is too short to be antigenic, it may be conjugated to a carrier molecule to enhance the antigenicity of the peptide. Examples of carrier molecules, include, but are not limited to, human albumin, bovine albumin and keyhole limpet hemo-cyanin ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr A.I. (eds) Appleton and Lange, Norwalk Connecticut, San Mateo, California).

Exemplary antibody molecules for use in the detection methods of the present invention are intact immunoglobulin molecules, substantially intact immunoglobulin molecules or those portions of an immunoglobulin molecule that contain the antigen binding site, including those portions of immunoglobulin molecules known in the art as F(ab), F(ab'); $F(ab')_2$ and F(v). Polyclonal or monoclonal antibodies may be produced by methods known in the art. (Kohler and Milstein (1975) Nature 256, 495-497; Campbell "Monoclonal Antibody Technology, the Production and Characterization of Rodent and Human Hybridomas" in Burdon et al. (eds.) (1985) "Laboratory Techniques in Biochemistry and Molecular Biology," Volume 13, Elsevier Science Publishers, Amsterdam). The antibodies or antigen binding fragments may also be produced by genetic engineering. The technology for expression of both heavy and light chain genes in E. coli is the subject of the PCT patent applications: publication number WO 901443, WO 901443 and WO 9014424 and in Huse et al. (1989) Science 246:1275-1281.

The antibodies of this invention may react with native or denatured Sox-4 or TSEP-1 protein or peptides or analogs thereof. The specific immunoassay in which the antibodies are to be used will dictate which antibodies are desirable. Antibodies may be raised against the

either the Sox-4 or TSEP-1 protein or portions thereof or against synthetic peptides homologous to either the Sox-4 or TSEP-1 amino acid sequence.

In one embodiment the antibodies of this invention are used in immunoassays to detect either Sox-4 or TSEP-1 proteins in biological samples. In this method the antibodies of the present invention are contacted with a biological sample and the formation of a complex between either the TSEP-1 or Sox-4 protein and antibody is detected. Immunoassays of the present invention may be radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay, chemiluminescent assay, immunohistochemical assay and the like (In "Principles and Practice of Immunoassay" (1991) Christopher P. Price and David J. Neoman (eds), Stockton Press, New York, New York; Ausubel et al. (eds) (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, New York). Standard techniques known in the art for ELISA are described in Methods in Immunodiagnosis, 2nd Edition, Rose and Bigazzi, eds., John Wiley and Sons, New York 1980 and Campbell et al., Methods of Immunology, W.A. Benjamin, Inc., 1964, both of which are incorporated herein by reference.

The MHC Class I suppressing drugs which are administered according to this invention may be administered as a sterile pharmaceutical composition further comprising a biologically acceptable carrier including, but not limited to, saline, buffer, dextrose, ethanol and water.

The MHC Class I suppressing drugs which are administered may be administered alone or in combination with other drugs, hormones, or antibodies. Examples of drugs include, but are not limited to, MHC Class I suppressing drugs, immunosuppressive drugs, cytotoxic drugs and anti-inflammatory drugs. Examples of hormones include, but are not limited to, corticosteroids, steroids

and steroid derivatives, estrogens, androgens, growth factors such as insulin-like growth Factor I, glycoprotein hormones, cytokines and lymphokines. Examples of antibodies include, but are not limited to, antibodies directed against MHC Class I antigens, antibodies directed against MHC Class II antigens and antibodies against infectious antigens.

Means of administering the MHC Class I suppressing drugs include, but are not limited to, oral, sublingual, intravenous, intraperitoneal, percutaneous, intranasal, intrathecal, subcutaneous, intracutaneous, or enteral. Local administration to the afflicted site may be accomplished through means known in the art, including, but not limited to, topical application, injection, infusion and implantation of a porous device in which the MHC Class I suppressing drugs are contained.

A preferred means of administering the MHC Class I suppressing drugs in the treatment of autoimmune diseases and transplantation rejection is oral. A preferred means of pretreating tissues to be transplanted is by perfusion in vitro with an aqueous solution.

All books, articles, or patents referenced herein are incorporated by reference. The following examples illustrate various aspects of the invention but are no way intended to limit the scope thereof.

Example 1

Lack of Induction of Experimental SLE in MHC-Class I-Deficient Mice

Induction of Experimental SLE in Mice

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of an array of autoantibodies, among these are anti-DNA, anti-nuclear antigen, and anti-RNP antibodies (Talal, N. et al. (1977) Autoimmunity: Genetic, Immunology Virology and Clinical Aspects; Academic Press, NY). Progression of the disease in humans is correlated with leukopenia,

proteinuria, and immune complex deposits in the kidney and other organs. An experimental model of SLE can be induced in mice by immunization with a human monoclonal anti-DNA antibody expressing a common idiotype, designated 16/6Id. Following a single immunization and subsequent boost with the 16/6Id, mice produce antibodies to the 16/6Id, to DNA, and to nuclear antigens. After a period of 4-6 months, the immunized mice develop leukopenia and proteinuria, and immune complexes are observed in their kidneys (Mendlovic, S. et al, (1988) Proc. Natl. Acad. Sci. U.S.A., 85:2260-2264). This experimental model closely parallels the human disease with respect to the production of autoantibodies and to its clinical manifestations. Several other laboratories have used these antibodies to induce SLE in mice. The immunological basis for disease induction in 16/6Id-immunized mice is not known. Mice lacking cell-surface MHC class I molecules have been generated by inactivating the gene for β_2 microglobulin, which is required for the proper assembly and cell surface expression of the class I molecule (Zijlstra, M. et al., (1990) Nature, 344:742-746; Koller, B. et al., (1990) Science, 248:1227-1230; mice were provided by B. Koller). These Class I-deficient mice also fail to develop the $CD4^-$ $CD8^+$ T cell subset. Class I-deficient mice generally are healthy and capable of generating antibody responses and surviving various viral infections; however, they are more sensitive to intracellular parasites than their normal littermates. To determine whether class I molecules play any role in the induction or propagation of experimental SLE, class I-deficient mice were tested for their ability to develop this disease.

Mice (groups of 4-6; strain 129-class I deficient) were immunized intradermally into the hind footpads with 1 ug of affinity purified human monoclonal 16/6Id in complete Freund's adjuvant (CFA; Difco, Detroit, MI) and boosted 3 weeks later with 1 ug of 16/6Id in

phosphate-buffered saline (PBS) (Mendlovic, S. et al, (1988) Proc. Natl. Acad. Sci. U.S.A., 85:2260-2264). Immunization of strain 129-Class I-deficient mice with chicken ovalbumin (Grade v. sigma Chem. Co. St. Louis, MO) was at 20ug and followed the same regimen as 16/6Id.

Analysis of Anti-16/6Id and Anti-DNA Antibodies In Class I-Deficient Animals

In Class I^+ control strain 129 mice (Jackson Labs, Bar Harbor, Maine), anti-16/6Id and anti-DNA responses were detected in the sera by ELISA. ELISAs were performed using 16/6Id and anti-16/6Id as described (Mendlovic, S. et al., (1988), Proc. Natl. Acad. Sci. (USA) 85:2260-2264; Heineman, W.R. et al (1987) Methods Of Biochemical Analysis 32:345-393). Anti-16/6Id and anti-DNA responses were detected within 10 days post-boost and persisted for at least 6 months; results are shown from animals 10 weeks after the boost (Figure 1A and 1B). Sera from 16/6Id-immunized animals did not contain significant anti-human immunoglobulin reactivity. Class I-deficient mice immunized with the 16/6Id developed anti-16/6Id antibodies at the same time as, and with titers not significantly different from, the control strain 129 mice (Figure 1A). In contrast, sera of the Class I-deficient mice did not contain significant anti-DNA antibody (Figure 1B); no significant anti-DNA response was detected in the class I-deficient animals for up to at least 6 months. During this time, anti-16/6Id titers remained high in the sera of both class I-deficient and control strain 129 animals. Furthermore, anti-nuclear antigen antibodies were not detected in sera of class I-deficient animals, but were found in sera of 16/6Id-immunized strain 129 animals. (Figure 1C). The class I-deficient mice were not generally poor responders to antigen, as immunization with ovalbumin elicited an antibody response not markedly different from that of normal mice (Figure 1D).

It has previously been reported that C57BL/6 mice are non-responders to the 16/6Id (Mendlovic, S. et al., (1990) Immunology, 69:228-236). Since the C57BL/6 mice failed to generate anti-16/6Id antibodies, this non-response is distinct from that of the class I-deficient mice which made anti-16/6Id antibodies, but no anti-DNA or anti-nuclear antigen antibodies. Furthermore, C57BL/6 X Class I-deficient F1 mice responded normally to the 16/6Id.

Response of Class I-Deficient Animals to Immunization With Monoclonal Anti- 16/6Id Antibody

The development of anti-DNA antibodies in normal mice immunized with 16/6Id is correlated with the generation of anti-anti-16/6Id antibodies (Mendlovic, S. et al., (1988) Proc. Natl. Acad. Sci. U.S.A. 85:718-730); immunization with anti-16/6Id triggers antibodies to DNA and nuclear extract, and experimental SLE (Mendlovic, S., et al., (1989) Eur. J. Immunol, 19:2260-2264). The failure of Class I-deficient mice to develop anti-DNA antibodies in response to immunization with 16/6Id raised the possibility that they do not respond to anti-16/6Id. This possibility was assessed by immunizing class I-deficient mice with murine monoclonal anti-16/6Id.

Mice (groups of 6) were immunized in the hind foot pads with 20 μg of monoclonal, anti-16/6Id 1A3-2 (Mendlovic, S. et al., (1989) Eur. J. Immunol, 19:729-734) in CFA and boosted 3 weeks later with the same amount of monoclonal antibody in PBS. Mice injected with a control anti-Id antibody (Mendlovic, S. et al. (1989) Eur. J. Immunol 19:729-734) did not develop a response. Although the control strain 129 mice all responded to the anti-16/6 idiotype, the class I-deficient mice did not respond at all (Figures 2A-2D). Thus, class I-deficient mice are capable of responding to ovalbumin and 16/6Id, but they are defective in their response to anti-16/6Id antibody (Figures 1A-1D and 2A-2D).

Analysis of Leukopenia, Proteinuria and Immune Complex Disease in Normal and Class I-Deficient Mice Immunized with 16/6Id

Immunization of control 129 mice with 16/6Id not only elicited an extended antibody response, but also induces leukopenia, proteinuria, and immune complex disease in the kidney (Table 1, Fig. 3A). Since Class I-deficient mice did not mount the full range of antibody responses following 16/6Id immunization, their susceptibility to these clinical manifestations of disease was monitored. Whole blood was collected from the tail vein of the mice into heparin diluted 1:10 in PBS, followed by a 1:10 dilution in 1% acetic acid in distilled water to lyse the red blood cells. Leukocytes were then microscopically counted in a hemocytometer using conventional methods. Protein levels in urine were measured by conventional colormetric assays on Ames 2855 Uristix (Miles, Inc.). None of the Class I-deficient mice showed any evidence of either leukopenia or proteinuria (Table I). Assessment of immune complex disease in the kidney of control and Class I-deficient animals was determined by immunohistology using frozen kidney sections, 5 μm thick, fixed and stained with FITC-conjugated goat antimouse IgG as previously described (gamma chain specific: Sigma Immunochemicals St. Louis, MO.; Fricke H. et al. (1991) Immunology, 73:42-427). Immune complex deposits were readily detected in the kidneys of 16/6Id-immunized control mice; no such deposits were found in the kidneys of class I-deficient animals (Figure 3B). Taken together, these data indicate that class I-deficient mice do not develop experimental SLE.

Table 1

Class I-Deficient Mice Immunized With 16/6Id
Do Not Develop Clinical Manifestations of SLE

Animals	Treatment	Leukocyte Counts (#/mm^3)	Proteinuria (mg/dL)
Experiment #1:			
129	None	5500±250	Negative
129	16/6Id	3150±50	100
Class I-deficient	None	5500±250	Negative
Class I-deficient	16/6Id	5530±250	Trace
Class I-deficient	Ovalbumin	5130±155	Negative
Experiment #2:			
129	None	5500±3000	Negative
129	16/6Id	2680±135	30-100
Class I-deficient	None	5500±250	Negative
Class I-deficient	16/6Id	4480±193	Negative-Trace
Class I-deficient	Ovalbumin	4033±88	Negative

Legend to Table 1. Five or six months after immunization, blood was drawn from class I-deficient and control 129 mice. Leukocyte counts were performed on each individual animal. The results represent the mean±SEM. The leukocyte counts of the 16/6Id immunized class I-deficient and control 129 animals are significantly different ($p<0.002$); those of the class I-deficient mice immunized with 16/6Id or ovalbumin are not significantly different ($p<0.2$), and fall within the normal range. Protein in the urine was measured using an Ames 2855 Uristix (Miles, Inc.); normal mice were negative.

Example 2

MMI as a Therapeutic Drug in SLE Mice

As described in Example 1, to induce experimental SLE, Balb/c mice were immunized intradermally with human monoclonal anti-DNA antibody, 16/6Id, in complete Freund's adjuvant and boosted 3 weeks later with 16/6 Id in saline. Anti-16/6Id antibodies could be detected in all mice within two weeks of the boost (Figure 4A). After two weeks, mice were treated with a subcutaneous injection of MMI in pellet form, which results in a 30 day release of the drug. The pellet in these experiments contained 15 mg MMI (0.5 mg released per day; Innovative Research of America, Toledo, Ohio). Treatment was repeated 30 days later. Several groups of 16/6Id immunized mice were evaluated: mice treated with MMI alone, with MMI plus thyroxine (1.5 mg/pellet, 30 day release, Innovative Research of America, Toledo, Ohio) to prevent hypothyroidism, or with a MMI placebo (Innovative Research of America, Toledo, Ohio). In addition, normal mice that had not been immunized with 16/6Id were treated with an identical drug regimen. Mice were bled at regular intervals and monitored by various parameters. Serum was assayed for the presence of anti-16/6Id antibodies and anti-DNA antibodies by ELISA, as described in Example 1. Peripheral blood cells were counted and analyzed for expression of various cell surface markers, including MHC class I and class II, by flow cytometry using labelled specific antibodies which are commercially available. In addition, protein in the urine was measured as described in Example 1. Finally, after 6 months, mice were sacrificed and kidneys analyzed for immune complex deposits. Immunohistology was performed as described in Example 1.

Effect of MMI on the formation of anti-16/6Id and anti-DNA Antibodies

Within two weeks of the 16/6Id boost, both anti-16/6Id and anti-DNA antibodies were detected in all of the immunized mice (Figures 4A-4B). In untreated, control mice, the anti-16/6Id antibody titers increased for 4-8 weeks post boost and the anti-DNA antibody titers increased for four weeks post boost; both persisted for the duration of the experiment. MMI treatment of 16/6Id immunized mice resulted in a small but reproducible decrease in the level of anti-16/6Id antibody titre over the 4 month post treatment period (Table II-A, Figure 4C). Anti-DNA antibody titers were markedly lower in MMI-treated than untreated 16/6Id immunized animals (Table II-B; Figure 4D). Treatment with thyroxine (T_4), together with MMI, partially reversed the decrease in anti-16/6Id antibody titers (Table II; Figure 4C) but did not significantly affect anti-DNA antibody titers (Table 2; Figure 4D). Placebo alone caused a modest inhibition in antibody titers, but never as much as that of MMI. Taken together, these data demonstrate that MMI treatment caused a decreased generation of anti-DNA antibodies.

TABLE II

A. Relative Anti-16/6Id Antibody Titers

Rx[a]	0 weeks	3 weeks	4 weeks	7 weeks	9 weeks	12 weeks	22 weeks
16/6Id	1.0	1.0	1.0	1.0	1.0	1.0	1.0
16/6Id + MMI	0.77	0.71	0.74	0.86	0.68	0.65	0.54
16/6Id + MMI+T_4	0.82	0.71	0.97	0.93	1.0	0.97	0.83
16/6Id + placebo	-	-	0.89	0.94	0.83	-	-
MMI	0.0	0.0	0.0	-	0.0	0.05	0.0
MMI+T_4	0.0	0.0	0.0	-	0.0	0.05	0.0
Placebo	-	-	0.01	-	0.0	-	-

[a]The column designated Rx indicates treatment received by animals.

TABLE II

B. Relative Anti-DNA Antibody Titers

Rx[a]	0 weeks	3 weeks	4 weeks	7 weeks	9 weeks	12 weeks	22 weeks
16/6Id	1.0	1.0	1.0	1.0	1.0	1.0	1.0
16/6Id MMI	2.18	0.67	0.16	0.11	0.17	0.18	0.0
16/6Id + MMI+T_4	2.18	0.67	0.16	0.11	0.17	0.18	0.0
16/6Id + placebo	-	-	0.53	0.49	0.46	-	-
MMI	0.14	0.08	0.03	0	0.03	0.16	0.0
MMI+T_4	0.14	0.18	0.038	-	0.19	0.0	-
Placebo	-	-	0.08	-	0.15	-	-

[a]The column designated Rx indicates treatment received by animals.

Effect of MMI on the development of leukopenia and proteinuria

A characteristic feature of this SLE model, is that mice treated with the 16/6Id antibody develop leukopenia as a function of time and as one of the clinical manifestations of the developing disease. (Figure 5). Mice immunized with 16/6Id and treated with MMI did not develop leukopenia (Figure 5). The effect of MMI was not prevented by simultaneous treatment with thyroxine (Figure 5) nor was it duplicated by placebo treatment. The protective effect of MMI persisted at least 4 months after MMI treatment was discontinued. Furthermore, proteinuria, which is a clinical manifestation in 16/6Id immunized mice, was prevented by MMI treatment.

Effect of MMI on the development of immune complexes in the kidney

After 4-6 months, mice immunized with 16/6Id developed immune complex deposits in the kidney which are associated with death due to renal failure (Figure 6, left). Kidneys were isolated from mice five months after MMI treatment ended, frozen and stained as described in Example 1. The pattern of immune complexes observed in the kidneys of 16/6Id immunized animals was similar to that in human kidneys derived from SLE patients (Figure 6A). MMI treatment of 16/6Id immunized mice markedly reduced the development of kidney lesions (Figure 6B). The effect of MMI is not prevented by simultaneous treatment with thyroxine nor was it duplicated by placebo treatment. The effect is evident for at least five months after MMI treatment.

Effect of MMI on lymphocyte populations during the course of the experimental disease

Although MMI has been used extensively in the treatment of autoimmune thyroid disease, its effect on various lymphocyte populations and cell surface expression

of MHC antigens has not been assessed previously. Since MMI has been shown to repress MHC class I transcription in vitro (Saji et al., 1992b) and because of its ability to mitigate the onset of experimental SLE, its effect on lymphocytes in vivo was evaluated.

According to the method described in Ehrlich, R. et al. (1989) Immunogenetics 30:18-26, peripheral blood lymphocytes (PBL) from 16/6Id-immunized mice, either MMI treated or not, were analyzed by flow cytometry for the proportion of T cells and B cells after MMI treatment . T cells were identified by their expression of the cell surface marker, Thy1, and B cells by their expression of B220 or MHC class II as detected by specific antibodies to these markers. Antibodies against these MHC Class I and MHC Class II surface markers, as well as others, are commercially available (Pharmingen, Boehringer - Mannheim; Erlich, R. et al. (1989) Immunogenetics 30:18-26). PBL from 16/6Id immunized mice consistently contained 15-20% B cells and 25-30% T cells (Figure 7A). The remainder being neither B cells nor T cells and are termed null cells (Figure 7A). This distribution did not vary markedly over the course of 6 months. Whereas MMI treatment had little or no effect on the proportion of T cells, it markedly reduced the fraction of B cells in the PBL (Figure 7A). There was a concomitant increase in the fraction of unstained cells. These changes in cell populations were most marked immediately after MMI treatment, but persisted for up to 2 months after MMI treatment had been discontinued. Thyroxine treatment, in conjunction with MMI, tended to partially reverse these effects.

The levels of MHC cell surface expression of the T and B cell populations were assessed by two-color flow cytometry (Ehrlich, R. et al. (1989)). PBL from 16/6Id treated animals did not express levels of MHC class I or class II significantly differently from non-immunized controls. MMI treatment resulted in a decrease in MHC

class I expression on the surfaces of both T cells and B cells (Figures 7B, 7C). In addition, MHC class II levels on B cells were also reduced (Figure 7D). These effects were most pronounced at early times after the 16/6Id boost and within one week after MMI treatment. As assessed by flow cytometry (Ehrlich, R. et al. (1989)), other cell surface markers were not affected by MMI.

Example 3

MMI as a Therapeutic Drug in NZB Mice

NZBxNZWF1 mice (Jackson Labs, Bar Harbor, Maine) spontaneously develop SLE (Steinberg, A.D. et al. (1990) Immunological Reviews 118:129-163; "Cellular and Molecular Immunology" (eds.) Abbas, Lichtman and Ruber (1992), page 360). These mice also spontaneously develop kidney lesions and produce anti-DNA autoantibodies.

NZBxNZWFI mice at six weeks of age, at which time there are no SLE symptoms, were started on MMI therapy. One 30 day MMI pellet (15 mg MMI) was injected subcutaneously every month as described in Example 1. Anti DNA antibodies in the serum were titered by ELISA monthly, as described in Example 1 and Example 2. As shown in Figure 8, MMI markedly decreased the anti-DNA titer after two months in this spontaneous disease model as in the 16/6Id model (Examples 1 and 2; Figs. 1A-1D, 2A-2D and 4A-4D). The effect of MMI on anti-DNA antibodies was even more pronounced three months after treatment.

Example 4

MMI as a Treatment for SLE in Humans

For treating humans suffering from SLE MMI is administered orally. Initially in a dose of up to 100 mg per day. This can be followed by a step-wise program, to 50 mg for up to 20 days, 40 mg for up to 20 days, 35 mg for up to 30 to 60 days, decreasing progressively to 5 mg - 30 mg per day. A maintenance dose of 5 mg - 10 mg per day for up to 1 year or longer can also be used. TSH levels can be monitored to assess the therapeutic levels

of MMI required for the SLE patient. When TSH levels increase significantly above the normal range, MMI dosage can be decreased to the next dose level. Alternatively, thyroid hormone levels can be used to determine dosage changes of MMI. A significant decrease from the normal range can be used as an indication to lower dosage. Since patients can be treated with thyroid hormone (T_4 or T_3) plus MMI to maintain a euthyroid state, the TSH level is a better index. The same parameters may be assessed in children.

Patients can be monitored for alleviation of clinical signs and symptoms of active disease. Specifically monitored parameters can include, autoantibodies, particularly DNA antibodies, PBL cell surface markers, leukopenia, proteinuria, hyperimmunoglobulinemia and levels of immune complexes in the kidney by punch biopsy.

Example 5

In Vitro Treatment Of FRTL-5 With Methimazole For Transplantation Into Wistar Rats or Balb/c Mice

Rat FRTL-5 (American Type Culture Collection, Rockville, MD; CRL 8305; US 4,609,622; US 4,608, 341) cells were grown to near confluency in complete 6H medium and then exposed, or not, to methimazole, 5 mM, for 72 hours in the presence of the normal complete 6H medium (Saji et al. (1992b). Cells from 4 plates were then harvested by trypsinization as per cell transfer, scraping, or with cold HBSS (Hanks Balanced Saline Solution) plus EDTA, 2mM. Cells were centrifuged as for splitting cells, resuspended in complete medium, recentrifuged and suspended in 0.1 to 0.2 ml medium. Cells were then injected subcutaneously, in the lower back, into normal Balb/c mice (NIH; Jackson Labs, Bar Harbor, Maine) or Wistar rats (NIH; Jackson Labs, Bar Harbor, Maine). Sixty days later, cells from the site of injection were isolated by surgical excision of the entire

implantation site and exposed to a mixture of collagenase trypsin, and chicken serum (CTC; Kohn, L.D. et al. U.S. Patent No. 4,609,622 Ambesi-Impiombato U.S. Patent No. 4,608,341; Kohn, L.D. and W.A. Valente, FRTL-5 Today, (eds) F.S. Ambesi-Impiombato and H. Perrild (1989):244-273)) to isolate individual cells, then plated in Petri dishes in normal 6H medium. Thyroid cell presence was evaluated microscopically; however, in all cases cells were cultured to confluency, subcultured in 24 well plates in 6H medium, then maintained 5 days without TSH before measuring TSH-induced iodide uptake or TSH-induced cAMP levels (Kohn et al., U.S. Patent No. 4,604,622). The increase induced by TSH was compared to control cells not treated with TSH. Thyroid cells (FRTL-5) were found only in cultures from the site of injection in which cells were pretreated with MMI (Table III). The cultures containing these thyroid cells also exhibited TSH-increased cAMP levels and TSH increased iodide uptake (Table III). In contrast, cultures from the site of injection in which the FRTL-5 cells had not been pretreated with MMI contained only fibroblast cells (Table III). In addition, no TSH increased cAMP levels or TSH increased iodide update were observed. These results show that pretreatment of FRTL-5 cells with MMI prevents rejection after transplantation. Simultaneously cultured FRTL-5 cells were positive controls. Four animals were in each group. The experiment was repeated with similar results.

TABLE III

	No MMI	Plus 5 mM MMI
Balb/c mice		
Microscopy	Fibroblasts only	Thyroid cells plus fibroblasts
TSH increased cAMP	No	Yes (8 ± 3 fold)
TSH increased iodide uptake	No	Yes (5 ± 1.5 fold)
Wistar rats		
Microscopy	Fibroblasts only	Thyroid cells plus fibroblasts
TSH increased cAMP	No	Yes (10 ± 3 fold)
TSH increased iodide uptake	No	Yes (6 ± 1 fold)

In a second experiment, two animals each were given cells which had been treated with 0.2% serum and 3H (no insulin, hydrocortisone, TSH) for 6 days plus or minus MMI for 72 hours. As evaluated by microscopy no animal had thyroid cells after 60 days and none had a TSH response in either assay. This would be expected since MMI action appears to require serum and since class I is maximally expressed under these conditions, in vitro, in the absence of serum, insulin, hydrocortisone and TSH (Saji et al. (1992(b)).

In a third experiment, FRT rat thyroid cells, a line of cells with no TSH receptor mRNA and no thyroid function (Ambesi-Impiombato F.S., Coon H.G. (1979) Int Rev Cytol Suppl. 10:163-171; Akamizu T, et al., (1990) Proc. Natl. Acad. Sci. USA, 87:5677-5681), were permanently transfected with human TSHR cDNA using a neomycin selection procedure (Van Sande J. et al., (1990) Mol. Cell. Endocrinol, 74:R1-R6). The transfected FRT thyroid cells were treated with, as were the FRTL-5 cells, 5 mM MMI for 72 hours and transplanted into the backs of Balb/c mice as described above. Sixty days later cells were isolated and shown to have a TSH-increased cAMP response as described above. Control cells with transfected TSHR

cDNA which were not treated with MMI or control FRT cells with no TSHR cDNA, when similarly implanted and evaluated, did not exhibit a TSH-increased cAMP level. This indicates that a transfected gene can survive the MMI procedure to transplant cells.

Example 6

Assessment of the Effect of MMI on MHC-Class I Expression by Gel Shift Assay

Materials

Purified bovine TSH was from the NIH program (NIDDK-bTSH-I-1, 30U/mg) or was prepared as described previously (Kohn, L.D. and Winand, R.J. (1975) J. Biol. Chem., 250:6503-6508). Insulin, hydrocortisone, human transferrin, somatostatin, glycyl-L-histodyl-L-lysine acetate were from (Sigma Chemical Co. St. Louis, MO). [^{125}I] cAMP radioimmunoassay kits, [α-^{32}P]dCTP (3000 Ci/mmol) and [^{32}P]UTP (3000 Ci/mmol) were from Du Pont/New England Nuclear (Boston, MA).

Cell Culture

FRTL-5 rat thyroid cells (Kohn LD. et al., US Patent no. 4,609,622; Ambesi-Impiombato ES., US Patent no. 4,608,341) are grown as described. These cells do not proliferate in the absence of TSH, yet remain viable for prolonged periods in its absence. Their doubling time was approximately 36 ± 6 hours; and, after 6 days in medium with no TSH (5H) and 5.0% serum, $1x10^{-10}$ mol/L TSH elevated iodide uptake 8-10 fold and thymidine incorporation > 10 fold. Cells were diploid, between their 5th and 25th passage in most experiments, and were routinely grown in Coon's modified F12 medium supplemented with 5% calf serum, 1 mmol/L nonessential amino acids (GIBCO) and a mixture of 6 hormones (6H medium): TSH ($1X10^{-10}$ mol/L), insulin (10 mg/L), hydrocortisone 1(nmol/L), human transferrin (5 mg/L), somatostatin (10 μg/L) and glycyl-L-

histidyl-L-lysine acetate (10 μg/L) (Kohn, L.D. et al. U.S. Patent No. 4,609,622; Ambesi-Impiombato, E.S. U.S. Patent No. 4,608,341). They were passaged every 7-10 days and provided fresh media every 2 or 3 days. In individual experiments, cells were shifted to medium with no TSH (5H), to medium with neither TSH and or insulin (4H), or to medium with no TSH, no insulin, and no hydrocortisone (3H) plus either 5% or 0.2% serum for 4-6 days before use.

Cell extracts

Cells were grown in 6H medium with 5% calf serum medium for 6-7 days to 70-80% confluence, then shifted to 5H medium with 5% calf serum for 5 days. TSH ($1x10^{-10}$ M) and/or MMI (5mM) were added as appropriate for 40-44 hours. Cells were then harvested and extracts were made by a modification of a method of Dignam, J. et al. (1983) Methods in Enzymology, 101:582-598. In brief, cells were harvested by scraping after being washed twice with cold phosphate-buffer saline (PBS). Subsequently they were pelleted, washed in cold PBS and then pelleted again. The pellet was resuspended in Dignam buffer C (20 mM Hepes buffer at pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 25% glycerol, 0.5mM dithiotreitol, 0.5 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin). The final NaCl concentration was adjusted on the basis of cell pellet volume to 0.42 M and cells were lysed by repeated cycles of freezing and thawing. Extracts were then centrifuged at 10,000 xg at 4°C for 20 min. The supernatant was recovered, aliquoted and stored at -70°C.

Gel Mobility Shift Assay

Binding reactions were performed in a volume of 20 μl for 30 min at room temperature. The typical reaction mixture contains 1.5 fmol of ^{32}P DNA, 3 μg of cell extracts, 3μg of poly (dI-dC) in 10 mM Tris-Cl (pH 7.9), 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol. Unlabeled competitor (a 100- to 1000-fold excess of double-stranded oligonucleotides or 200-fold excess of PD1

promoter fragments) was added to the appropriate control binding reactions 20 min before the ^{32}P to insure specificity. After incubation, reaction mixtures were subjected to electrophoresis in 4% polyacrylamide gels for 90-120 min at 160 V in 0.5x TBE (Sambrook, J., et al., (1989) then dried and autoradiographed. Probes were labeled by Klenow enzyme (In Vitro labeling kit, Amersham), following manufacturer instructions, and then purified through G-50 columns (5 Prime→3 Prime).

Positive and negative regulatory (enhancer or silencer regions, respectively) elements have been identified in the promoter of the swine MHC class I gene, PD1 (Singer and Maguire (1990)). The activity of these enhancers and silencer regions is mediated by trans-acting factors (Singer and Maguire (1990) Cirt. Rev. Immunol. 10:235-257). Two regulatory domains have been identified in the 5' flanking region of the PD1 gene. One regulatory domain is between approximately -1 and -300 bp from the transcriptional start site. This region contains an interferon response element and a major enhancer, as well a site homologous to a cyclic AMP response element (CRE) element. Studies using gel mobility shift assays have demonstrated that TSH/CAMP-induced or modified proteins interact with this region and can regulate transcription initiation (Saji et al. (1992a)). Another complex regulatory region, showing overlapping silencer and enhancer activity, has been mapped between -690 and -769 base pairs upstream of the promoter (Weissman, J.D. and Singer, D.S. (1991) Mol. Cell. Biol. 11:4217-4227). The enhancer and silencer elements are linked to tissue specific expression and tissue specific levels of the Class I gene (Weissman, J.D. and Singer, D.S. (1991)).

The Saji et al (1992b) study showed reduced expression of MHC Class I gene in rat FRTL-5 cells treated with MMI. This study also showed that the effect of MMI in MHC Class I expression was at the level of

transcription. The FRTL-5 thyroid cell system is therefore a good system to identify the regulatory DNA sequence elements and trans-acting factors involved in the MMI effect. PD1 Gel shift mobility assays were performed using the 5' flanking region of the PD1 gene and cell extracts from FRTL-5 cells treated with MMI, TSH and MMI plus TSH.

Figures 9A-9B shows the sequence of the PD1 promoter with the 151 (bases 54 to 220 of SEQ ID NO:1), 114 (bases 221 to 320 of SEQ ID NO:1), 140 (bases 321 to 455 of SEQ ID NO:1) and 238 (bases 456 to 692 of SEQ ID NO:1) regions of the 5' portion of the PD1 promoter (SEQ ID NO:1) designated as indicated (Weismann, J.D. and Singer, D.S. (1991)). Figure 10 shows the silencer and enhancer regions of the 140 region (SEQ ID NO:2) with oligonucleotides used to map the region for the activity of the gel shifts. The silencer region of relevance is noted by the opposite arrows separated by a TTF-2 like, insulin-sensitive element. Figure 11 shows the alignment of the 114 (SEQ ID NO:36), 140 (SEQ ID NO:37), and the 105 (SEQ ID NO:35) region of the 238 region of the PD1 promoter to show sequence homology. The silencer region is indicated by arrows separated by TTF-2 like region. These fragments were derived from the PD1 promoter of the PDI Class I MHC gene (Singer D.S. et al. (1982) Proc. Natl. Acad. Sci. USA, 79:1403-1407).

Figures 12A-12D show gel shifts using the radiolabelled 140 (bases 321 to 455 of SEQ ID NO:1) (Figures 12A and 12D), 114 (bases 221 to 320 of SEQ ID NO:1) (Figure 12B) and 151 (bases 54 to 220 of SEQ ID NO:1) (Figure 12C) fragments noted in Figure 9. The complex affected by MMI is denoted A. In Figure 12A, 12B and 12C, lane 4 shows the complex formed between the silencer region (see Figure 10 and below) and cell extracts from FRTL-5 rat thyroid cells maintained in the presence of 5H medium (no TSH) plus 5% serum. The effect

of the addition of 5 mM MMI for 24 hours prior to extract preparation from cells maintained in 5H medium is shown in lane 5 of Figures 12 A-D. The effect of the addition of $1x10^{-10}$M TSH for 24 hours prior to extract preparation from cells maintained in 5H medium is shown in lane 6 in Figures 12 A-C. The effect of the addition of 5 mM MMI plus $1x10^{-10}$M TSH for 24 hours prior to extract preparation from cells maintained in 5H medium is noted in lane 7 in Figure 12A and 12B. The effect of the addition of $1x10^{-10M}$ TSH for 7 days before extracts were prepared (6H, MMI-) is noted in lane 3 in Figure 12A-C. The effect of the addition of $1x10^{-10}$M TSH plus 5mM MMI for 24 hours before extracts were prepared (6H, MM1+) is noted in lane 2 in each case. Lane 1 in Figures 12 A-C contains the radiolabelled probe alone. The ability of 200-fold excess concentration of unlabeled 151 fragment (bases 54 to 220 of SEQ ID NO:1) to compete A complex formation with the 151 radiolabelled fragment (bases 54 to 220 of SEQ ID NO:1) is shown in lane c, Figure 12C. Competition to inhibit MMI-sensitive A complex formation by 200-fold higher concentrations of unlabeled 105 (bases 588 to 692 of SEQ ID NO:1) (lane a, Figure 12C), 140 (bases 321 to 455 of SEQ ID NO:1) (lane b, Figure 12C) and 114 (bases 221 to 320 of SEQ ID NO:1) (lane d, Figure 12D) are noted showing that the A complex formed with each complex is the same. In panel D, lane e shows the basal A complex formed between the silencer region (see Figure 10 and below) and cell extracts from FRTL-5 rat thyroid cells maintained in the presence of a 3H medium plus 0.2% calf serum. In contrast to cells maintained in the 5H plus 5% serum case (Figure 12(A)), MMI (lane f), TSH (lane g) or both together (lane h) added to cells for 24 hours does not significantly affect A complex formation in 3H medium (Figure 12D). 3H medium has no insulin as well as no TSH. The ability of 200-fold excess concentration of unlabeled 105 (bases 588 to 692 of SEQ ID NO:1) (lane i) to inhibit

formation of the MMI-insensitive A complex in 3H medium shows that same complex appears to be involved, but the absence of insulin and/or serum in 3H medium prevents the TSH and MMI inhibitory effect. The lack of A complex formation in the absence of the 3H cell extracts is noted in lane j.

Figure 14(A) shows gel shifts using the radiolabelled 238 fragment (bases 456 to 692 of SEQ ID NO:1) noted in Figure 9 and cell extracts from FRTL-5 rat thyroid cells maintained in the presence of a 5H hormone mixture (no TSH) plus 5% serum (5H Basal) Lane 2). The complex affected by MMI is denoted A; inhibition of the formation of this complex by cellular extracts from FRTL-5 cells treated for 24 hours with 5 mM MMI plus $1x10^{-10}$M TSH is noted in lane 14. The 238 construct (bases 456 to 692 of SEQ ID NO:1) encompasses the 105 construct (bases 588 to 692 of SEQ ID NO:1) (see Figure 9); complex A forms with the 105 portion (bases 588 to 692 of SEQ ID NO:1) of the 238 (bases 456 to 692) construct as evidenced by the ability of a 200-fold excess concentration of unlabeled 105 (bases 588 to 692 of SEQ ID NO:1) over radiolabelled 238 (bases 456 to 692 of SEQ ID NO:1) to inhibit complex A formation (lane 3). The A complex in lane 2 is formed between the silencer region (see Figure 10 and above) and is the same as that formed with the 114 (bases 221 to 320 of SEQ ID NO:1), 140 (bases 321 to 455 of SEQ ID NO:1), and 151 (bases 54 to 220 of SEQ ID NO:1) constructs (Fig. 12) as evidenced by the following. First, a 200-fold higher concentration of unlabeled 114 (bases 221 to 320 of SEQ ID NO:1) (lane 4) and 140 (bases 321 to 455 of SEQ ID NO:1) (lane 5), compared to radiolabelled 238 (bases 456 to 692 of SEQ ID NO:1), inhibited A complex formation; a 200-fold higher concentration of 151 (bases 54 to 220 of SEQ ID NO:1) was a partial inhibitor (lane 6). Second, a 1000-fold concentration of double stranded oligonucleotide with the sequence of the silencer region (S2 (SEQ ID NO:4)

in Figure 10), relative to radiolabelled 238, inhibited A complex formation; the same concentration of double-stranded oligonucleotide mimicking the sequence of the enhancer element (E1 (SEQ ID NO:20) in Figure 10) had no effect on A complex formation. Oligonucleotides with modifications of the silencer sequence (S1 (SEQ ID NO:3), S3 (SEQ ID NO:10), S6 (SEQ ID NO:6), S7 (SEQ ID NO:7), and S8 (SEQ ID NO:8) in Figure 10) were partial inhibitors at the 1000-fold concentration (lanes 9-13). The inhibition by S1 (SEQ ID NO:3) (lane 12) suggested that mutation of only one of the end repeats denoted by the arrows in Figure 10 is enough to decrease inhibition; the partial inhibition by S8 (SEQ ID NO:8) (lane 10) suggested that the element which resembles the sequence reactive with TTF-2 in the thyroglobulin promoter (Santisteban, P., et al., and Mol. Endocrinol. 6:1310-1317, 1992) and that is between the inverted repeats (Figure 10) is also important in formation of the A complex. This conclusion is supported by the result in lane 7. The presence of a 1000-fold concentration of the K oligonucleotide (SEQ ID NO:38) which mimics the sequence of the thyroid transcription factor-2 (TTF-2)-reactive element in the thyroglobulin promoter (Santisteban, P. et al., Mol. Endocrinol. 6:1310-1317, 1992) enhanced A complex formation and by the result in lane 15 which showed that a 1000-fold higher concentration of unlabeled oligonucleotide K (SEQ ID NO:38) was able to reverse the MMI/TSH action. Thus, decreased formation of the MMI-sensitive A complex requires TTF-2 and insulin, consistent with the data in Figure 12D. The K oligonucleotide (SEQ ID NO:38) "ties up" insulin-induced TTF-2 which results in increased complex formation and loss of the MMI effect, i.e. there is a requirement for insulin.

Figure 14(B) further demonstrates the importance of TTF-2 to the MMI action and provides an additional means to assay the MMI effect. Figure 14(B) shows gel

shifts using the radiolabelled K oligonucleotide (TGACTAGCAGAGAAAACAAAGTGA) (SEQ ID NO:38) and cell extracts from FRTL-5 rat thyroid cells maintained in the presence of a 5H hormone mixture (no TSH) plus 5% serum (5H Basal) (Lane 16). The upper FRTL-5 cell protein/DNA complex formed is inhibited by treating cells for 24 hours with 5 mM MMI (lane 17), with $1x10^{-10}$ TSH (lane 18) and with 5 mM MMI plus $1x10^{-10}$M TSH (lane 19). The TTF-2 upper protein/DNA complex is therefore necessary for MMI action and important in A complex formation noted in Figure 14A. Inhibition of its formation is a means to assay the MMI effect and supports the insulin-dependency of MMI action.

The complexes detected below the A complex in Figures 12 A-D and Figure 14 A-B are believed to be enhancer complexes (uppermost bands below the A complex) or nonspecific complex. The intense signal at the bottom of the autoradiographs in Figures 12 A-D and Figure 14 A-B was unbound probe.

Taken together these results suggest that inhibition of complex formation can be used as an indicator of MMI or other drugs to down regulate MHC Class I transcription.

The A complex is believed to be composed of different proteins. The different proteins are important in determining the level of tissue specific complexes between tissues. TSH induced the formation of a new thyroid specific complex in the -200 to -1 region of the PD1 promoter. This complex was also increased by 5 mm MMI and involved a TTF-2-like transcription factor. This complex was increased as the A complex decreases. Its formation was associated with TATAA box activity. We propose this thyroid specific protein/DNA complex dominates the tissue-specific silencer/enhancer complex (Figure 10) and decreases gene expression by decreasing the initiation of transcription of the Class I gene.

Example 7

Assessment of the Effect of MMI on MHC Class I Expression by CAT Assay

Plasmid construction, DNA probes and oligonucleotides

The full length PD1 promoter, PD1 CAT construct pH(-38), inserted into the multicloning site of pSV3CAT, has been previously described (Erhlich, R. et al. (1989) Immunogenetics 30:18-26). Sequential deletion mutants of the full length PD1 promoter, inserted into the multicloning site of pSV3CAT, have been previously described (Singer and Weismann (1991); Saji et al (1992a); Saji et al. (1992b)). Briefly, a nested series of 5' deletions of the upstream regulator region of the PD1 gene were generated by Bal31 digestion; the series 5' termini ranged from -1012 base pairs to -68 base pairs; all had a common 3' boundary at +15 base pairs. The deletion series was also cloned into the pSV3CAT reporter construct to assess promoter activities (Singer and Weisman (1991); Maguire, J. et al. (1992) Mol. Cell. Biol 12:3078-3086).

Figure 13 shows transfection data with chloramphenicol acetyltransferase (CAT) chimeras showing that MMI inhibits full length PD1 promoter activity.

Rat FRTL-5 thyroid cells were put in fresh 6H medium containing 5% calf serum 12 hours before transfection by the electroporation method described previously (Saji et al 1992 b). In brief, FRTL-5 cells were grown to 80% confluence, harvested, washed, and suspended at 1.5×10^7 cells/ml in 0.8 ml electroporation buffer (272 mM sucrose, 7 mM sodium phosphate at pH 7.4, and 1 mM $MgCl_2$). Twenty μg of the full length CAT construct were added with 5 μg pSVGH. Cells were then pulsed (330 volts, capacitance 25 μFD), plated (approximately 6×10^6 cell/dish), and cultured for 12 hours in 6H medium containing 5% calf serum medium. At that time, cells were placed in 5H medium plus 5% calf serum (control), 5H medium plus 5% calf serum plus 5 mM MMI

(MMI+), 6H medium plus 5% calf serum (TSH+), or 6H medium plus 5% calf serum plus 5 mM MMI (MMI/TSH). After 40 hours they are harvested. Cell viability was approximately 80%. Medium was taken for hGH radioimmunoassay to monitor transfection efficiency. (Nichols Institute, San Juan Capistrano, CA) and cells were harvested for CAT assays which used 20-50 μg cell lysate in a final volume of 130 μl. Incubation was at 37°C for 2 or 4 hours; acetylated chloramphenicol was separated by thin layer chromatography (TLC) and positive spots on TLC plates were cut out and quantitated in a scintillation spectrometer. Data are expressed as the ratio of CAT activity to GH activity. The full length PD1 promoter includes the 151 (bases 54 to 220 of SEQ ID NO:1), 114 (bases 221 to 320 of SEQ ID NO:1), 140 (bases 321 to 455 of SEQ ID NO:1), and 238 (bases 456 to 692 of SEQ ID NO:1) regions (Fig. 9). As shown in Figure 13 treatment with MMI (), TSH and MMI () and TSH () decrease CAT activity relative to the control (). CAT activity of the chimeric CAT constructs of the sequential deletion mutants can also be used on CAT assays to assay the effect of MMI on Class I promoter activity. CAT activity is, therefore, another way to assay the effect of MMI on class-I promoter activity and can be used for evaluating other agents able to mimic MMI in therapeutic actions related to treatment of autoimmune disease or transplantation therapy.

Example 8

Identification Of Transcription Factors Which Regulate The Upstream Silencer/Enhancer And The Effect Of MMI/TSH On These Factors

Materials and Methods

Materials. TSH and other hormones are the same as in Example 6. MMI and insulin were from the Sigma Chemical Co. (St. Louis, MO); rabbit polyclonal antibodies against the p50 and p65 subunits of NF-κB, c-fos family

members, and c-jun/AP1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [α-^{32}P]deoxy-CTP (3000 Ci/mmol) and [^{14}C]chloramphenicol (50 mCi/mmol) were purchased from DuPont-New England Nuclear (Boston, MA); [γ-^{32}P]ATP (6000 Ci/mmol) was from Amersham (Arlington Height, IL). Calf serum was a heat-treated, mycoplasma free product from GIBCO Laboratories Life Technologies, Inc. (Grand Island, NY). The source of all other materials was the Sigma Chemical Co., unless otherwise noted.

Cell Culture. FRTL-5 rat thyroid cells (Interthyr Research Foundation, Baltimore, MD; ATCC No. CRL 8305) were a fresh subclone (F1) with all the properties previously detailed (Example 6; Saji, M., et al., (1992a)). Fresh medium was added every 2 or 3 days and cells were passaged every 7-10 days. In individual experiments, cells were shifted to medium with no TSH (5H medium) or with no TSH, no insulin, plus 0.2% serum (4H medium) for 6 to 8 days; other agents were added as noted.

Plasmid construction, DNA probes and oligonucleotides. The full length PDI promoter chimera encoding 1100 bp of the 5' flanking region of the MHC class I PD1 swine promoter, linked to a chloramphenicol acetyl-transferase (CAT) reporter gene, has been described as have chimeras with sequential deletion mutants of the -1100 bp class I sequence (See Example 7); Weissman, J. D. and Singer, D. S. (1991) Mol. Cell. Biol. 11, 4217-4227; Giuliani, C., et al., (1994) J. Biol. Chem. 270, 11453-11462; Ehrlich, R., et al., (1988) Mol. Cell Biol. 8, 695-703; Maguire, J. E., et al., (1992) Mol. Cell. Biol. 12, 3078-3086; Howcroft, T. K., et al., (1993) EMBO J. 12, 3163-3169). The series 5' termini ranged from - 1100 to -89 bp; all had a common boundary at +15 bp. The numbering of different chimeras is determined from +1, the start of transcription, and extends to the numbered nucleotide (Guiliani, C. et al. (1995) J. Biol. Chem. 270:1453-11462 herein incorporated by reference. The start of

transcription is nucleotide 1091 in Figure 9. After addition of XbaI linkers, the PD1 fragments were subcloned into the XbAI/Hind III sites of PSV3CAT, which has a multicloning site at the NdeI site in pSV0CAT. Other CAT constructs were created by polymerase chain reaction using 100 pmol each of an appropriate forward primer with a BamHI site on the 5'-end and an antisense reverse primer of the PD1 sequence from -13 to +1 bp of the transcription start site which had a HindIII site on the 3'-end. Mutants of p(-127)CAT and p(-89)CAT were created by two-step, recombinant PCR methods (Saiki, R. K., et al (1988) Science 239, 487-491; Higuchi, R. (1990) In: *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand D. H., Sninsky, J. I., and White T.J. eds) Academic Press, Inc., San Diego, 177-183). In the first step, two PCR products that overlap the sequence were created, both of which contain the same mutation introduced as part of the PCR primers. The second step PCR was performed using these overlapped PCR products as template and DNA sequence of the 5' or 3'-end of the final products as primer. The PCR products were inserted into the multicloning site of pSV3CAT as above or pCAT-enhancer-less and pCAT control vectors purchased from Promega (Madison, WI). In the case of the pCAT vector, the CRE-like sequence and its mutants were created with a BamHI site on both ends of the primers. The pSV0-based constructs containing the CAT gene downstream of different lengths of the 5'-flanking region of the swine class I (PD1) gene which were used herein are termed p(-1100)CAT, p(-400)CAT, p(-294)CAT, p(-203)CAT, p(-127)CAT, and p(-89)CAT; numbered from the nucleotide at the 5'-end to +1 bp, the start of transcription.

DNA probes for the PD1 promoter regions used herein were obtained as previously reported (in Example 6; Weissman, J. D. and Singer, D. S. (1991) Mol. Cell. Biol. 11, 4217-4227; Giuliani, C., et al., (1994) J. Biol. Chem.

270:11453-11462; Ehrlich, R., et al., (1988) Mol. Cell Biol. 8, 695-703; Maguire, J. E., et al., (1992) Mol. Cell. Biol. 12, 3078-3086; Howcroft, T. K., et al., (1993) EMBO J. 12, 3163-3169). Double-stranded oligonucleotides containing the sequences of the silencer and enhancer which control constitutive MHC class I levels in different tissues, and oligonucleotides with mutations of these sites, were those described (Weissman, J. D. and Singer, D. S. (1991) Mol. Cell. Biol. 11, 4217-4227). Similarly, double-stranded oligonucleotides containing the sequence of the insulin responsive elements (IREs) of the thyroglobulin (TG) and TSH receptor (TSHR) promoters, oligo K and TIF, respectively, were synthesized as described (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Shimura, Y., et al., (1994) J. Biol. Chem. 269, 31908-31914). Oligo K or oligo TIF were also annealed and inserted in pUC19 plasmids for transfection experiments. Briefly, pUC19 plasmids were linearized with XbaI, dephosphorylated with alkaline phosphatase, and ligated to the blunt-ended oligonucleotides using T4 DNA ligase.

After purification, plasmids were sequenced (Sanger, F., et al., (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467) to insure directional fidelity and confirm copy number. All plasmid preparations were twice purified by CsCl gradient centrifugation (Davis, L. G., et al., (1986) *Basic Methods in Molecular Biology*. Elsevier, New York, 93-98).

Transfection

FRTL-5 cells stably transfected with class I promoter-CAT chimeras have been described (Giuliani, C., et al., (1995) J. Biol. Chem. 270:11453-11462). To test the effect of TSH or MMI, cells were grown to 70-80% confluency in 6H medium, then maintained without TSH (5H medium) for 5 days, at which time they were exposed to $1x10^{-10}$ M TSH or 5 mM MMI for 40 hours before CAT activity

was measured. Transient transfections using the same class I-CAT chimeras were performed by electroporation (Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 793-804; Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 1701-1715) using one of two procedures. FRTL-5 cells maintained in 6H medium were transfected and 12 hours later were treated one of three ways: 5 mM MMI was added with fresh 6H medium; 6H medium was replaced by fresh 5H medium; or cells were maintained in fresh 6H medium. Alternatively, FRTL-5 cells were maintained without TSH (5H) for 5 days and were returned to medium with TSH (6H) for 12 hours before transfection. They were then plated for 12 hours in 6H and the medium then changed to 5H medium plus or minus 5 mM MMI and plus or minus TSH as noted.

For electroporation (Gene Pulser, BioRad, Richmond, CA), the procedure was the same as in Example 7. and as described (Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 793-804; Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 1701-1715) with the following exceptions. Either 20 μg p(-1100)CAT or equivalent molar amounts of the deletion mutants or pSV0CAT (negative control) were used; these amounts were determined in preliminary experiments which optimized transfection conditions as a function of plasmid concentration. After 36-44 hours, cells were harvested and CAT activity measured (Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 793-804; Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 1701-1715; Gorman, C. M., et al., (1982) Mol. Cell Biol. 2, 1044-1051) using 20 μg cell lysate and an incubation at 37°C for 4 hours. Acetylated chloramphenicol was separated by thin layer chromatography and autoradiographed; positive spots were excised and quantitated in a scintillation spectrometer.

A DEAE-dextran procedure (Lopata, M. A., et al., (1984) Nucleic Acids Res. 12, 5707-5717) was used to cotransfect 20 μg each of chimeric class I promoter-CAT constructs and pUC19 plasmids with or without oligo K,

oligo TIF, or their respective mutants. Cells were grown to 80% confluency in 6H medium, shifted to 5H medium 12 hours before transfection, washed twice with phosphate buffered saline, pH 7.4 (PBS), and incubated 1 hour with 5 ml serum-free 5H medium containing the plasmid DNA plus 250 μg DEAE-dextran (5 Prime-3 Prime, Inc.). Cells were then exposed to 10% dimethylsulfoxide in PBS for 3 min., washed twice in PBS, cultured in 5H medium for 12 hours, then maintained therein another 36 hours with or without MMI or TSH as noted. CAT assays were performed as above. (See also Example 7).

Efficiency of transfection was determined by cotransfection with 5 μg pRSVLuc, kindly provided by Dr. S. Subramani, U. of CA, LaJolla. CAT values, mean ± S.E. of 3 experiments, are normalized to luciferase activity and protein using the Promega assay system and a Monolight 2010 luminometer. Cell viability was approximately 80% in all experiments.

Extracts. Cell extracts were made by a modification of a described method (Dignam, J., et al., (1983) Nucleic Acids Res. 11, 1475-1489) as described in Example 6 with the following additions or exceptions. In same experiments cells were grown in 6H medium until 80% confluent and then maintained in 5H medium (-TSH) with 5% calf serum or 4H medium (-TSH, -insulin) with only 0.2% serum for 7 days. Experiments were initiated by exposing cells to $1x10^{-10}$M TSH or 5 mM MMI. Pelleting was by centrifugation at 500g, after being washed twice in cold PBS, ph 7.4. The pellet was resuspended in 2 volumes of Dignam buffer C (Dignam, J., et al., (1983) Nucleic Acids Res. 11, 1475-1489; see Example 6) and the final NaCl concentration was adjusted on the basis of cell pellet volume to 0.42 M. Cells were lysed by repeated cycles of freezing and thawing. The extracts were centrifuged at 35,000 rpm (100,000xg) and at 4°C for 20 min. The supernatant was recovered, aliquoted, and stored at -70°C.

To perform gel mobility shift or Western blot assays using nuclear extracts and involving multiple experimental points, i.g. experiments performed as a function of time, a rapid and efficient technique for extraction of nuclear proteins from one or two culture dishes was used. The method modifies a method to isolate hemopoietic cell nuclei (Bunce, C.M., et al. (1988) Anal. Biochem. 175, 67-73) then extracts proteins from the nuclei with a high salt buffer (Henninghausen, L., et al., (1987) Methods in Enzymology 152, 721-735). In this procedure all samples and reagents are kept on ice. For centrifugation a microcentrifuge is used with its maximum speed setting. Buffer A and Buffer B contain 0.5 mM DTT, 0.5 mM pMSF, 2ng/ml pepstatin A and 2 ng/ml Leupeptin. Typically, 5 X 10^5 or more cells are washed with 10 ml of Dulbecco's modified phosphate buffered saline without Mg^{2+} and Ca^{2+} (DPBS), pH 7.4, scraped and collected in a microcentrifuge tube with 1 ml of DPBS. Cells are pelleted by centrifugation for 30 seconds at room temperature, resuspended in five volumes of 0.3M sucrose, 2% Tween 40 in Buffer A (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA) frozen in dry ice-ethanol, and kept at -80°C, if desired, for further analysis. The cells are thawed in a 37°C water bath and, using a micropipet with a yellow tip, pipetted 50 to 100 times (depending on the number of cells or volume of the samples) to release nuclei. Samples are overlayed on 1 ml of 1.5 M sucrose in Buffer A and centrifuged for 10 minutes at 4°C. Nuclei are pelleted to the bottom of the tube, cytoplasmic organelles and cell membrane debris are located in the intermediate phase. The nuclear pellets are washed with 1 ml of Buffer A by centrifugation for 30 seconds, and then resuspended in 10 ml of Buffer B (20 mM HEPES KOH, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0,2 mM EDTA, 25% glycerol). Samples are placed on ice for 20 minutes with occasional vortexing, followed by centrifugation for

20 minutes at 4°C. The supernatant fraction, containing nuclear protein is aliquoted and stored a -70°C. Optimal pipetting to disrupt the cell membrane and release nuclei before sucrose centrifugation is important to get good results. Before and after this sucrose centrifugation, the purity of nuclei and/or distribution of other cellular components is able to be determined by observing samples under a phase contrast microscope with trypan blue staining. .

Electrophoretic Gel mobility Shift Assays (EMSA)

PD1 promoter probes were obtained by restriction enzyme digestion as previously reported (Examples 6 and 7; Weissman, J. D. and Singer, D. S. (1991) Mol. Cell. Biol. 11, 4217-4227; Giuliani, C., et al., (1995) J. Biol. Chem. 270:11453-11462; Ehrlich, R., et al., (1988) Mol. Cell Biol. 8, 695-703; Maguire, J. E., et al., (1992) Mol. Cell. Biol. 12, 3078-3086; Howcroft, T. K., et al., (1993) EMBO J. 12, 3163-3169) and were purified from 2 % agarose gels using a QIAEX extraction kit (Quiagen, Chatsworth, CA). They were labeled with [α-^{32}P]dCTP using Klenow, whereas oligo K was radiolabeled with [γ-^{32}P]ATP using T4 kinase. Radiolabeled probes were purified by electrophoresis on an 8% native polyacrylamide gel for 1-2 hours at 120 V (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Binding reactions with cell extracts were carried out in a volume of 20 μl for 30 min at room temperature; reaction mixtures contained 1.5 fmol of [^{32}P]DNA, 3 μg cell extract, and 3.0 μg poly(dI-dC) in 10 mM Tris-Cl (pH 7.9), 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ethylenediamine tetraacetic acid (EDTA), 5% glycerol, and KCl as indicated in some experiments. Where indicated, unlabeled double-stranded oligonucleotides were added to the binding reaction as competitors and incubated with the extract for 20 min

prior the addition of labeled DNA. Similarly, in supershift experiments using antisera, extracts were incubated in the same buffer containing either immune or normal rabbit serum at room temperature for 20 min before adding labeled DNA. Following incubations, reaction mixes were subjected to electrophoresis on 4 or 5 % native polyacrylamide gels for 1-2 hours, at 160 V, in 0.5xTBE, and at room temperature. Gels were dried and autoradiographed.

Transcription Extension Assays. In vitro transcription extension (run-on) assays were performed as described (Saji, M., Moriarty, et al., (1992) J. Clin. Endocrinol. Metab. 75, 871-878; Isozaki, O., et al., (1989) Mol. Endocrinol. 3, 1681-1692). Aliquots of the purified [^{32}P]UTP-radiolabeled nuclear RNA were hybridized with excess amounts of the class I, TG, and ß-actin cDNA inserts or control pSG5 (Stratagene) or pBR322 (New England Biolabs) plasmid DNA immobilized on nylon membranes.

Sox-4 Cloning And Recombinant Sox-4 Protein

To clone rat Sox-4, a λgt11 FRTL-5 thyroid cell cDNA expression library (Akamizu, T., et al., (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5677-5681) was screened by a modification of the Southwestern blotting procedure (Vinson, C. R., et al., (1988) Genes Dev. 2, 801-806) using a polymerized oligonucleotide with 8 repeats of the thyroglobulin insulin response element (oligonucleotide K; TGACTAGTAGAGAAAACAAAGTGA). In the primary screen, the library was plated at a density of 40,000 plaque-forming units/143 cm^2. After 4 h at 42°C, the plates were overlaid with nitrocellulose filters that had been soaked in 10 mM isopropyl ß-D-thiogalactopyranoside (IPTG) and were then incubated for 12 h at 37°C. The nitrocellulose filters were removed from the culture plates and allowed to air dry for 15 min at room temperature. Dried filters, with bound protein, were denatured by platform shaking for 10

min at 4°C in binding buffer (10 mM Tris-HCl, pH 7.6, 200 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol) containing 6 M guanidine hydrochloride. After repeating this step, the denaturing solution was diluted with an equal volume of binding buffer without guanidine hydrochloride and the shaking continued for 5 minute at 4°C. Filters were subjected to 4 consecutive 5 minute washings, each with a two-fold dilution of the guanidine hydrochloride, two 5 min washes with unsupplemented binding buffer, and then transferred to a blocking solution containing 5% Carnation non-fat dry milk in binding buffer. After gentle shaking for 30 min, the filters were exposed to $1x10^6$ cpm ^{32}P-labeled DNA probe in binding buffer containing 50 μg/ml poly(dI-dC), 20 μg/ml denatured calf thymus DNA, 0.62 mM $ZnSO_4$, and 0.25% dry milk for 1 hour at room temperature. Subsequently, filters were washed 3 times for 10 minute in binding buffer containing 0.25% dry milk at 4 °C before autoradiography. The probe used for screening was generated by concatenating the annealed and phosphorylated oligonucleotide with T4 ligase. Ligated products were isolated by agarose gel electrophoresis, and cloned into the blunt-ended XbaI site of the pCAT-Promoter plasmid (Promega, Madison, WI). As needed, the DNA fragment containing eight repeats of oligo K was isolated from a stock plasmid, nick-translated, and used as a probe for screening. The cloned cDNA was ligated to the EcoRI site of PUC19, and sequenced as described (Isozaki, O., et al., (1989) Mol. Endocrinol. 3, 1681-1692 41). Sequence alignments and comparisons were performed using PC-GENE and GENE WORKS software (IntelliGenetics, Mountain View, CA).

To obtain purified recombinant protein, a NcoI-EcoRI fragment (-1 to 1411 bp) of rat SOX-4 cDNA was ligated between the NcoI and EcoRI sites of pET30a(+) (Novagen, Madison, WI). The recombinant protein, whose N-

terminus was fused to a consecutive stretch of 6 histidine residue, was produced in the bacterial strain BL21(DE3). A single colony was inoculated in 50 ml LB medium containing 30 μg/ml kanamycin and incubated with shaking at 37°C. At 0.6 OD_{600}, isopropyl-ß-D-thiogalactopyranoside (IPTG) was added to 1 mM. After 3 h of induction by 1 mM IPTG, cells were collected by centrifugation (5,000xg, 5 min, 4°C), resuspended in 4 ml ice-cold binding buffer (5 mM imidazole, .0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), then sonicated until no longer viscous. Affinity purified protein was obtained using Ni^{2+} charged resin (Novagen, Madison, WI). Cell extracts were centrifuged (39,000xg, 20 min, 4°C); the supernatant was applied to His-Bind columns containing resin-immobilized Ni^{2+}; and the columns were washed with 25 ml binding buffer. Unbound proteins were removed with 15 ml wash buffer; Sox-4 was recovered with 15 ml elute buffer containing imidazole. The His-Bind column contained 5 ml resin and was washed, sequentially, with 7.5 ml deionized water, 12.5 ml charge buffer (50 mM $NiSO_4$) and 12.5 ml binding buffer. After addition of a 1/3rd volume of Strip Buffer, the eluted fraction was dialyzed against 20 mM HEPES-KOH, pH 7.9, 100 mM KCl, 0.1 mM EDTA, 20 % glycerol, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, and 2 μg/ml pepstatin A, then concentrated in a Centricon 10 (Amicon, Beverly, MA) for use in electrophoretic mobility shift assays (EMSA).

Other Procedures and Statistical Significance

Protein concentration was determined by Bradford's method (BioRad) and used recrystallized bovine serum albumin as the standard. All experiment were repeated at least three times with different batches of cells. Values are the mean ± S.E. unless otherwise noted. Significance between values was determined using two-way analysis of variance; values were significant if P values were <0.05.

Results

In run-on assays, MMI and TSH treatment of FRTL-5 thyroid cells, maintained in medium containing insulin (5H) plus 5% serum, independently and additively decrease the transcription rate of class I genes (Figure 15A; consistent with Figure 13 in Example 7). The ability of MMI and TSH to decrease class I transcription requires the presence of the insulin and/or serum in the medium. Thus, MMI and TSH, alone or together, lose their ability to decrease class I transcription rates when examined using nuclei from cells maintained 7 days without insulin and with only 0.2% calf serum in the medium (Figure 15B). These data suggested that the transcriptional suppression of class I by MMI not only involves factors which are additively and independently regulated by TSH, but also factors regulated by insulin and/or components of the serum. The TSH action can be duplicated by stimulating TSH receptor autoantibodies in Graves' IgG preparations (data not shown).

At the same concentrations which are maximally effective in run-on assays (Figure 15A), MMI and TSH independently and additively decrease the activity of a chloramphenicol acetyltransferase (CAT) chimera containing 1100 bp of class I 5'-flanking region, p(-1100)CAT, which had been transfected transiently into FRTL-5 thyroid cells (Figures 16 And 16B). Thus, MMI and TSH additively and independently regulate exogenous as well as endogenous class I promoter activity in the thyrocytes. The TSH action can again be duplicated by stimulating TSH receptor autoantibodies in Graves' IgG preparations.

To further localize the regulatory elements where MMI might act, MMI's effect on a series of 5' deletion constructs of the 1100 bp class I swine promoter-CAT chimeras stably (Table IV) or transiently (Figures 16A-16B) transfected into FRTL-5 cells. As shown in thyroid as well as nonthyroid cells (Figures 16A; Table

IV; Weissman, J. D. and Singer, D. S. (1991) Mol. Cell. Biol. 11, 4217-4227; Giuliani, C., et al., (1995) J. Biol. Chem. 270:1453-11462; Ehrlich, R., et al., (1988) Mol. Cell Biol. 8, 695-703; Maguire, J. E., et al., (1992) Mol. Cell. Biol. 12, 3078-3086; Howcroft, T. K., et al., (1993) EMBO J. 12, 3163-3169), 5'-deletions between -1100, -400, -294, and -203 bp increased class I promoter activity, indicating the presence of a series of negative regulatory elements between -1100 to -203 bp (Figure 16A). Deletion to -127 bp decreased, whereas truncation to -89 bp increased promoter activity (Figure 16A). These data are consistent with the existence of Enhancer A in the interval -203 to -127 bp (Ting and Baldwin (1993) Current Opinion in Immunol. 5:8-16) and a constitutive silencer between -127 and -89 bp (Example 9). MMI and TSH additively decreased exogenous promoter activity in all the chimeras tested (Table IV, Fig. 16A). Since activity persisted in a chimera within 89 bp of initiation of transcription, the function of elements below -89 bp were affected by both agents. These data did not, however, exclude the possibility that MMI/TSH regulated the activity of proteins which interacted with upstream elements and that their function was linked to elements within -89 bp of the start of transcription. This possibility was supported by two observations.

TABLE IV

Effect of 5 mM MMI or $1x10^{-10}$M TSH on the exogenous promoter activity in FRTL-5 cells stably transfected with chimeric CAT constructs of the 5'-deletion mutants of the swine class I promoter. The CAT activity of each chimera with no treatment is set at the control value to which the effect of treatment is compared (% of control).

CHIMERA	NO TREATMENT CONTROL	+ TSH ($1x10^{-10}$M)	+MMI (5 mM)
		% of Control	*% of Control*
p(-1100)	100	60 ± 7	63 ± 8
p(-400)	100	67 ± 6	75 ± 7
p(-294)	100	73 ± 5	78 ± 5
p(-203)	100	50 ± 5	54 ± 7
p(-127)	100	50 ± 7	55 ± 4
p(-89)	100	63 ± 6	68 ± 5
pSV0	100	100 ± 7	110 ± 7

Figure Legend for Table IV. FRTL-5 cells were grown to near confluency in 6H medium (plus TSH) and were maintained in 5H medium (no TSH) for 7 days before being treated with TSH or MMI for 40 hours. Control cells were those maintained in 5H medium for the same 40 hours. CAT activity was measured as described (Example 7; Example 8, Materials and Methods). The MMI or TSH treatment decreased CAT activity significantly ($P<0.05$ or 0.01) in cells transfected with all the CAT plasmids except the pSV0 control. Figure 16B notes the structure of each chimeric CAT construct used.

First, the additive effect of MMI and TSH decreased the exogenous class I promoter activity of p(-1100)CAT to levels of the pSV0 control (Fig. 16A). This paralleled their additive ability to decrease class I RNA levels (Saji et al. (1992b) J. Clin. Endocrin. Metabol. 75:871-878), complex formation with Class I promoter sequences (Example 6), and run-on assays (Figure 15) to comparable minimal levels. Deletion to -400 bp eliminated the additive ability of both agents to decrease promoter activity toward that of the pSV0 control; however, this phenomenon returned in the p(-127)CAT chimera, after deletion of the region between -203 and -127 bp, and was lost again in the -89 bp CAT chimera. Maximally additive TSH/MMI activity on the exogenous promoter, which matched maximal TSH/MMI-induced decreases in endogenous class I gene expression, appeared, therefore, to be associated with the activity of the silencer elements lost in the deletions between -1100 to -400 bp and -127 to -89 bp.

Second, as shown in Example 9, the function of the silencer between -127 to -89 bp depends on an octomer sequence, -107 to -100 bp (Figure 9), with homology to known cAMP-response elements (CREs). (Figure 9) (Saji, M., et al., 1992 a, 1992b; Montminy, M. R., et al., (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6682-6686; Angel, P., et al., (1987) Mol. Cell. Biol. 7; 2256-2266; Leonard, J., et al., (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6247-6251; Vallejo, M., et al., (1992) J .Biol. Chem. 267; 12868-12875; Leonard, J., et al., (1993) Mol. Endocrinol. 7, 1275-1283; Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 1701-1715; Habener, J. F. (1990) Mol. Endocrinol. 4, 1087-1094). Additionally, it is shown in Example 9 that TSH/cAMP treatment of FRTL-5 cells induces the appearance of new protein/DNA complex with the CRE-like element of the 38 bp silencer, whose formation is prevented by elements within the class I promoter region between -89 to

+1 bp (Example 9). Two of the proteins interacting with the downstream silencer, a thyroid transcription factor-1 and a Y-box protein, designated TSEP-1 (TSHR suppressor element protein-1), also interact with elements within +89 to +1 bp (See Figure 43). These data suggested TSH/MMI might, in fact, modulate the activity of factors interacting with the downstream silencer, as well as elements below -89 bp.

In the remainder of Example 8, the ability of MMI/TSH to additively and independently modulate the activity of the upstream silencer between -724 to -697 bp is characterized. In Example 9, the ability of MMI/TSH to modulate the activity of the downstream silencer (-127 to -89 bp) is shown. In addition, it is shown they are interactive, that the MMI/TSH action on each requires factors regulated by insulin and/or serum, albeit different factors, and that the MMI/TSH effect on the downstream silencer is functionally dominant. (Example 8, 9,10 and 11).

The silencer element between -724 and -697 bp has been shown to function together with an overlapping enhancer element to regulate constitutive levels of class I expression in different tissues (Example 6; Weissman, J. D. and Singer, D. S. (1991) Mol. Cell. Biol. 11, 4217-4227). By comparison to extracts from control FRTL-5 cells maintained in 5H medium alone (Fig. 12A, lane 4, 5H Basal), treatment of the cells with MMI and TSH (Fig. 12A, lanes 5 and 6, respectively) for 24 hours decreased the formation of a protein/DNA complex (arrow A) with a class I promoter fragment including residues between -770 and -636 bp. This fragment is termed the 140 Fragment (Figures 9 and 11) and it includes both the upstream silencer and its overlapping enhancer (Figure 10). The MMI/TSH effect was additive (Fig. 12A, lane 7). Moreover, TSH treatment of cells for 6 days caused a greater decrease (Fig. 12A, lane 3) than TSH treatment for 24

hours (Fig. 12A, lane 6); but MMI treatment for 24 hours was still additive (Fig. 12A, lane 2 vs 3).

The TSH/MMI-induced decrease in complex formation with the 140 Fragment required insulin/serum, consistent with the functional requirement for insulin/serum on TSH or MMI action in run on assays (Figs. 15A-15B). Thus, TSH and MMI treatment of FRTL-5 cells did not decrease formation of the A complex in FRTL-5 cells maintained in medium without insulin and plus only 0.2% serum (Fig. 12D). Formation of the complex was specific, as evidenced by self competition with a 200-fold excess of the unlabeled 140 Fragment.

The 140 Fragment encompasses both a silencer and an overlapping enhancer (Fig. 10). The MMI/TSH sensitive complex at the top of the gel (Figs. 12A-12D, complex A) appeared to be the silencer, based on its mobility and the prominence of the complex (Example 6; Weissman, J. D. and Singer, D. S. (1991) Mol. Cell. Biol. 11, 4217-4227). Thus, the silencer complex migrates near the top of gels (Weissman, J. D. and Singer, D. S. (1991) Mol. Cell. Biol. 11, 4217-4227); further, the high levels of silencer and low levels of enhancer complex are consistent with the low levels of class I expression found in thyroid cells (Saji, M., Moriarty, et al., (1992) J. Clin. Endocrinol. Metab. 75, 871-878; Saji, M., et al., (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1944-1948; Weissman, J. D. and Singer, D. S. (1991) Mol. Cell. Biol. 11, 4217-4227). The converse is true in tissues with high levels of expression, i.e. lymphocytes (Weissman, J. D. and Singer, D. S. (1991) Mol. Cell. Biol. 11, 4217-4227). To unequivocally establish that the MMI/TSH effect was on the silencer complex, we evaluated its formation in the presence of oligonucleotides able to inhibit formation of the silencer complex (Fig. 17A, S2 and S6), only the enhancer complex (Fig 17A, E9), or neither (Fig. 17A, S3) (Weissman, J. D. and Singer, D. S. (1991) Mol. Cell. Biol.

11, 4217-4227). As evidenced by inhibition by S2 and S6 but not S3 or E9 (Fig. 17A, lanes 2 and 4 vs 1, 3, and 5), complex a, whose formation is decreased by MMI and TSH (Fig. 17A), is the silencer. Complex b is the enhancer, as evidenced by its inhibition by E9 in the absence of an E9 effect on the silencer (Fig. 17A, lane 5).

Using unlabeled fragments from the class I promoter encompassing these related sequences, Fragment 105 (-503 to -399 bp), Fragment 114 (-870 to -770 bp), as well as Fragment 151 (-1036 to -871 bp), it was found that each was able to inhibit the formation of the silencer complex formed with the radiolabeled 140 Fragment (Fig. 17B, lanes 7 to 9 vs 6). Moreover, treatment of FRTL-5 cells with MMI and TSH, alone or together, inhibited the formation of a complex with identical mobility and in a manner similar to the silencer in the 140 Fragment, when each of these fragments were incubated with extracts from cells maintained in the presence of insulin and serum (Fig. 12B, lanes 1-7, compared to Fig. 12A, lanes 1-7) but not their absence (Fig. 12D). These results suggest that the silencer element exists at multiple loci between -1100 and -399 bp but that each is similarly modulated by TSH/MMI. This is consistent with the progressive increase in activity by 5' deletions of the class I promoter between -1100 and -294 bp and the progressive loss of the ability of MMI/TSH to decrease class I promoter activity to the levels of the pSV0 control. (Figure 16A)

Enhancer A of the Class I 5'-flanking sequence, -190 to -180 bp. is upstream of the interferon response element and CRE (Figure 16B). It is important for interferon action to increase Class I Levels and hydrocortisone actions to decrease them (Giuliani, C. et al. (1995) J. Biol. Chem. 270:11453-11462). A protein complex with enhancer A in thyroid cells is salt modulated termed Mod-1, the complex is regulated by hydrocortisone, insulin/serum, or interferon and includes the p50 subunit

of NF-κB and fra-2 (Giuliani, C., et al., (1995) J. Biol. Chem. 270:11453-11462). The silencer and enhancer complexes formed with the 140 Fragment and FRTL-5 cell extracts are salt sensitive (Fig. 18A). Thus, with increasing salt, it was noted that the silencer was composed of two separate protein/DNA complexes, both of which were modulated by TSH/MMI (data not shown). Using higher salt concentrations (100 μM KCL) in the shift assays, we could show that the lower complex appeared to be an adduct with the p65 subunit of NF-κB, as evidenced by the ability of an antisera to it, but not the p50 subunit of NF-κB, to supershift the lower complex to the level of, or a slightly higher level than, the upper complex (Fig. 18B, solid and dashed lines, respectively). The upper complex appears to be an adduct with a c-fos family member or a related protein (Fig. 18C). Thus, antisera reactive with c-fos family members caused a supershift (Fig. 18C, lanes 2 and 4), but not antisera specific for fra-1, fra-2, fosB, c-jun, or junB (Fig. 18C, lanes 5-9) nor a control normal serum (Fig. 18C, lane 3). These results, for the first time, identify protein components of the upstream silencer complex and support a relationship between it and Enhancer A.

The observation that MMI and TSH decreased, rather than increased, formation of the silencer complex was surprising, since the opposite might have been expected in association with increased silencer activity. An explanation for this and its role in MMI/TSH action on class I levels emerged from the following experiments. First, it was determined that the insulin/serum sensitivity of the MMI/TSH effect on the upstream silencer was related to a protein interacting with the insulin response element (IRE) of the thyroglobulin (TG) promoter, rather the insulin response element of the TSHR. Thus, *in vitro*, addition of oligo K, the oligonucleotide with the sequence of the TG-insulin response element, was able to

inhibit the decrease in complex formation with the upstream silencer evident in extracts from MMI/TSH-treated cells (Fig. 14A, lane 7 and 15). In contrast, oligo TIF of the TSHR insulin response element had no effect on complex formation by the upstream silencer (data not shown).

Second, it was determined that MMI/TSH action on the upstream silencer complex directly involved the protein interacting with the TG insulin response element. Thus, when oligo K itself was used as a radiolabeled probe, 2 major protein complexes were formed with cell extracts from FRTL-5 cells under the conditions employed (Fig. 14B, lane 16). Both were specific for oligo K as evidenced by competition using a 250-fold excess of unlabeled oligonucleotide, but not by a 250-fold excess of a mutant form of oligo K (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Aza-Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306) or 250-fold excess of oligo TIF (data not shown). More importantly, treatment of cells with MMI or TSH for 24 hours decreased the formation of the upper protein complex with oligo K and inhibited complex formation in an additive manner (Fig. 14B, lanes 17-19 vs 4, arrow A). The additive effect of MMI was evident in cells exposed to TSH (6H medium) for 6 days, exactly as in the case of the silencer complexes in the 140 Fragment. Further, the TSH/MMI modulated complex with oligo K required the presence of insulin/serum to form. Thus the complex was not present in extracts from cells maintained with no insulin and 0.2% serum (4H medium) and TSH/MMI had no apparent effect in the absence of formation of this complex.

Third, the protein binding the TG insulin response element and the silencer in the 140 Fragment of the class I promoter was required for upstream silencer activity, as well as the ability of MMI to decrease complex formation. Thus, cotransfection of a plasmid

containing oligo K and the p(-1100)CAT chimera of the class I promoter into FRTL-5 cells significantly increased promoter activity (Fig. 19A, oligo K1 and oligo K2). The increase was not duplicated by cotransfections with a mutant of oligo K (Fig. 19A, oligo KM), which loses its ability to compete for oligo K complexes with FRTL-5 cell extracts (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Shimura, Y., et al., (1994) J. Biol. Chem. 269, 31908-31914; Aza-Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306). The effect of oligo K was not duplicated when the p(-127)CAT chimera was used (Fig. 19B), consistent with the interpretation this effect was one involving the upstream not the downstream silencer. Thus, removal of the insulin-induced protein, by binding it to oligo K which had been transfected into the cells, functionally attenuated or eliminated silencer activity and resulted in the expression of enhancer activity.

Last, although the insulin-induced protein interacting with oligo K is necessary for silencer activity (Fig. 19) and the ability of MMI/TSH action to decrease silencer complex formation (Figure 14), its role in the MMI-decreased class I gene expression is dominated by the action of the insulin-induced protein interacting with the downstream silencer and the TSHR insulin response element. Thus, whereas oligo TIF cotransfection will be shown to cause a loss in the MMI effect on the p(-1100)CAT promoter chimera (Example 9), cotransfection with oligo K did not prevent the MMI decrease in p(-1100)CAT activity (Table V).

TABLE V

Effect of oligo K cotransfection on the ability of MMI/TSH to decrease Class I promoter activity of the p(-1100)CAT Chimera

CAT ACTIVITY (% of Control)

COTRANSFECTION	NO MMI	+ MMI
NONE	100 (Control)	*54 ± 8***
+ OLIGO K1	*155 ± 7**	*72 ± 12***
+ OLIGO K2	*214 ± 10**	*87 ± 11***
+ OLIGO KM	87 ± 9	*50 ± 6***

*Significant increase in activity, P<0.05 or better.
**Significant decrease in activity, P<0.05 or better, by comparison to activity in the absence of MMI.

Figure Legend for Table V. Using a DEAE-Dextran procedure, FRTL-5 cells grown in 5H medium plus 5% calf serum were cotransfected with p(-1100)CAT, as in Figure 16A, and 20 or 40 μg of a plasmid containing the oligo K oligonucleotide (Oligo K1 and Oligo K2, respectively) or 40 μg of a plasmid containing oligo KM, a mutated form of oligo K described previously (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Aza-Blanc, P., et al., (1993) Mol. Endocrino. 7., 1297-1306; Shimura, Y., et al., (1994) J. Biol. Chem. 269, 31908-31914) and used in Figure 19. Cells were maintained in medium with or without 5 mM MMI. CAT activity was measured 36 h later and conversion rates normalized to growth hormone levels; the activity of the control transfection with the p(-1100)CAT and the vector into which the oligoK sequences were inserted was assigned a value of 100%. Differences in the CAT activity of cells cotransfected with Oligo K or its mutant were compared to the control values. Values are the mean ± S.E. of three different experiments, each performed in duplicate.

The sum of these data suggested the following. The upstream silencer is decreased and it appears it must be disengaged to allow the downstream silencer to be engaged and functionally dominant. The p65 and c-fos complexes formed with the upstream silencer element exhibit silencer function only when they also interact with the insulin-induced protein able to bind to oligo K.

TSH/MMI action decrease upstream silencer complex formation by its action on the insulin-induced protein. Decreased upstream silencer complex formation is presumed to be associated with a loss of upstream silencer activity, but this may be a necessary accompaniment to MMI/TSH action on the downstream silencer, whose MMI/TSH-induced activity dominates. Thus, progressive deletions of the upstream silencer sites result in the attenuation of downstream silencer activity, whose dominance returns when all upstream silencer sites are deleted (Fig. 16A; Table IV).

The clone obtained and characterized herein which is the insulin-induced protein interacting with the upstream silencer and the TG-insulin response element, support the above hypothesis. These studies additionally show that the upstream enhancer, as well as the silencer, interacts with the TG-insulin response element reactive factor, which we designated as Sox-4.

A clone containing 1422 nucleotides whose open reading frame encodes a 442 amino acid residue protein with a molecular weight of 53,040 was obtained (Fig. 20A-20B). The protein is 98% similar to mouse Sox-4 (Van de Wetering et al. EMBO. Journal (1993) 12:3847-3854) and similar to human Sox-4 (Farr C.J. et al. (1993) Mammalian Genome 4:577-584). Mouse Sox-4 was cloned as a SRY related gene from T-lymphocytes which was responsible for transcriptional transactivation and can bind a sequence AACAAAG. Its function is not known (Van de Watering M. et al. (1993) EMBO J. 12:3847-3854). Rat and mouse Sox-4 are 32 residues smaller than human Sox-4 (Farr et al. (1993) Mammalian Genome 577-584). All of the extra residues in human Sox-4 and most of the different residues in mouse Sox-4 cluster within one region of the protein and are primarily glycine and alanine residues. It is not clear that this insert region in human Sox-4 is a neutral spacer region (Farr et al, (1993) Mammalian Genome 577-584),

since both mouse and human Sox-4 differences cluster in this region. The Sox-4 proteins are members of the HMG (high mobility group) class of transcriptional regulators, which bind DNA in a sequence specific fashion and include SRY, which regulates genes determining testicular development, TCF-1α, which regulates genes important in T cell development, and IRE-ABP (insulin response element A binding protein), which regulates genes subject to positive and negative regulation by insulin that appear to play a role in mediating the tissue specific effect of insulin on transcription of a diverse group of genes in lipogenic tissues (Alexander-Bridges M. et al. (1990) J. Cell. Biochem. 48:129-135). The common features of all three Sox-4 proteins include the HMG box (Figure 20, bold) and a serine-rich carboxy terminal tail with multiple putative casein kinase and histone kinase phosphorylation sites (Van de Wetering et al. (1993) EMBO Journal 12:3847-3854; Farr et al (1993) Mammalian Genome 577-584). The HMG box is a domain defined by its sequence similarity to HMG-1 and related proteins that associate with chromatin and are important in the structural organization of DNA. In the HMG class of transcriptional regulators, such as Sox-4, the HMG box exhibits sequence-specific DNA binding to the minor groove of DNA and induces a strong bend in the DNA helix (Van de Wetering et al. (1993) EMBO Journal 12: 3847-3854; Farr et al. (1993) Mammalian Genome 577-584; Ferrari et al. EMBO J. 11:4497-4506).

The Sox-4 recombinant protein described and characterized herein, can bind to oligo K, the TG insulin response element, or to a related oligonucleotide (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Francis-Lang, H., et al., (1992) Mol. Cell Biol. 12, 576-588; Aza Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306), oligo Z, which mimics the sequence of the related insulin response element on the thyroid peroxidase promoter (Fig. 21). Consistent with data reported in

competition studies, Sox-4 does not bind to the mutant of oligo K which loses its ability to interact with TTF-2, the presumptive binding factor which interacts with the TG insulin response element (Fig. 21) (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Francis-Lang, H., et al., (1992) Mol. Cell Biol. 12, 576-588; Aza Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306). The binding to the oligo Z mutant is also decreased, consistent with its decreased ability to compete for the TPO insulin response element in EMSA studies. Sox-4 binds weakly with oligo C of the TG promoter (Fig. 21), which mimics a site near the thyroglobulin (TG) or thyroid peroxidase (TPO) insulin response element that recognizes two different transcription factors: thyroid transcription factor 1 and Pax-8 (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Francis-Lang, H., et al., (1992) Mol. Cell Biol. 12, 576-588; Aza Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306; Civitareale, D., et al., (1993) Mol. Endocrinol. 7, 1589-1595; Civitareale, D., et al., (1989) EMBO J. , 2537-2542; Guazzi, S., et al., (1990) EMBO J. 9, 631-3639; Francis-Lang, H., et al., (1992) Mol. Cell. Biol. 12, 576-588; Zannini, M., et al., (1992) Mol. Cell. Biol. 12, 4230-4241; Shimura, H., et al., (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al., (1995) Endocrinology, 136, 269-282). These data are consistent with the oligonucleotide binding specificity ascribed to TTF-2, and alone might suggest Sox-4 is TTF-2.

However, although Sox-4 can footprint the region of insulin response element region of the TG promoter in DNAase-I protection experiments, the footprint is far more extensive than the TG insulin response element site ascribed to TTF-2 in FRTL-5 cell extracts. There is protection of the TG insulin response element, but the protection extends to the oligo C site which can bind TTF-1 and Pax-8 and to the oligo A region. This broad footprint is beyond that predicted in previous studies

defining TTF-2 (Santisteban, P. et al. (1992) Mol. Endocrinol. 6:1310-1317; Aza Blanc, P., et al. (1993) Mol. Endocrinol. 7, 1227-1306).

Northern analyses also do not fit a pattern consistent with Sox-4 being TTF-2. TTF-2 is a thyroid-specific transcription factor according to previous reports (Santisteban, P. et al. (1992) Mol. Endocrinol. 6:1310-1317; Aza Blanc, P. et al. (1993) Mol. Endocrinol. 7, 1297-1306); Civitareale, D., et al., (1993) Mol. Endocrinol. 7, 1589-1595; Civitareale, D., et al., (1989) EMBO J., 2537-2542; Guazzi, S., et al., (1990) EMBO J. 9, 631-3639; Francis-Lang, H., et al., (1992) Mol. Cell. Biol. 12, 576-588; Zannini, M., et al., (1992) Mol. Cell. Biol. 12, 4230-4241; Shimura, H., et al., (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al., (1995) Endocrinology, 136, 269-282). Thus, Northern analyses of Sox-4 with rat tissues confirm previous data with mouse or human Sox-4 (Farr, C. J., et al., (1993) Mammalian Genome 4, 577-584; van de Wetering, M., et al., (1993) EMBO J. 12, 3847-3854), i.e. they show Sox-4 is a ubiquitously expressed gene (Fig. 22A). Northern analyses further indicate that, although Sox-4 can be increased by insulin (Fig. 22B), as predicted for TTF-2 (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Francis-Lang, H., et al., (1992) Mol. Cell Biol. 12, 576-588; Aza Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306)., the increase requires more than 24 hours of insulin/serum treatment of FRTL-5 cells; the insulin-induced increase in oligo K complex formation with TTF-2 is, in contrast, already evident at 24 hours (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Francis-Lang, H., et al., (1992) Mol. Cell Biol. 12, 576-588; Aza Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306). Complex formation with TTF-2 is increased in extracts from cells treated with insulin or TSH after being maintained several days in 4H medium containing 0.2% serum, i.e. without

insulin, insulin-like growth factors, or TSH (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Francis-Lang, H., et al., (1992) Mol. Cell Biol. 12, 576-588; Aza Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306). This is true for Sox-4 (Fig. 22B). However, in the presence of insulin or serum, i.e. in a cell in 5H medium plus 5% serum, TTF-2 complex formation is not changed (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Francis-Lang, H., et al., (1992) Mol. Cell Biol. 12, 576-588; Aza Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306). In contrast, Sox-4 RNA levels are dramatically decreased by TSH in cells in 5H medium plus TSH for 1 week (Figure 22B, lane 6). Northern analyses do not, therefore, fit a pattern of RNA expression that might be expected for TTF-2 based on previous studies.

Overexpression of Sox-4 in FRT cells does not alter TG-CAT activity, whereas TTF-1 has been shown to have effects in cotransfection experiments. (Shimura, H. et al. (1994) Mol. Endocrin. 8, 1049-1069) Further, in FRTL-5 cells maintained in 6H plus 5% calf serum, where Sox-4 RNA levels are vanishingly low and where Sox-4/DNA complex formation is very low (Figure 22B), overexpression of Sox-4 can slightly increase TG-CAT activity on the full length promoter but has no effect on p.(-170) where the TG-IRE site is located (Table VI).

The properties of the Sox-4 protein identified herein do not fit several properties predicted for TTF-2 and is not TTF-2. Sox-4 is instead a component of the MHC class I upstream silencer/enhancer complex and regulates class 1 expression in the thyroid and other tissues.

Insulin increases complex formation with the upstream silencer and oligo K. In the presence of insulin, TSH decreases the upstream silencer complex (Fig. 12 and 14A) and decreases complex formation with oligo K. The basis for this is the ability of TSH to decrease Sox-4 RNA levels in the presence of insulin (Fig. 22B). The

basis for MMI action in Sox-4 is not an effect of MMI to decrease Sox-4 mRNA in the presence of insulin but altered thioredoxin activity (Table VII). Thus, Sox-4 activity may involve regulation by MMI through its action as a free radical scavenger (Wilson, R. et al. (1988) Clin. Endocrin. 28:389-397; Wienzel, N. et al. (1984) J. Clin. Endocrinol Metab. 58:62-69).

The silencer component regulated by Sox 4 is the p65 subunit of NF-kB (Fig. 18). Thus, antisera to Sox-4 supershifts the p65 upstream silencer complex (Fig. 23) decreasing silencer complex formation. The antisera also eliminates formation of the enhancer complex, which is composed of c-jun as evidenced by the ability of c-jun to form a similarly sized complex with the 140 fragment in the presence of Sox-4 or FRTL-5 cell extracts and an antisera to c-jun to inhibit enhancer complex formation (data not shown). Sox-4, therefore, is important in the formation of both the silencer and enhancer.

Sox-4 footprints a region overlapping both the enhancer and silencer (Fig. 24) and can suppress class I expression when a cDNA encoding Sox-4 is cotransfected with class I promoter-CAT chimeras (Table VI). This effect is specific since there is either no effect of the Sox-4 when cotransfected with thyroglobulin promoter-CAT chimeras or an increase in TG-CAT activity (Table VI).

The oligo K reactive protein important for class I regulation of the upstream silencer/enhancer is Sox-4. Sox-4 is a suppressor of class I as evidenced in cotransfection studies. In oligonucleotide (oligo K) transfection studies, oligo K which reacts with Sox-4, increases class I by blocking Sox-4 interactions with the silencer, rendering it nonfunctional, and increasing enhancer complex formation. TSH decreases Sox-4 mRNA levels and silencer complex formation, therefore one means to assess its action on Sox-4 is to evaluate Sox-4 by Northern analysis in addition to measuring silencer

complex formation. (Example 6). Another mechanism of measuring MMI action is to measure the increase Sox-4 activity modulated by effect of MMI on the actions of enzymes important in oxidation-reduction states of transcription factors, such as thioredoxin (Table VII) or superoxide dismutase (Wilson, R. et al. (1988) Clin. Endocrinology 28:389-397) via its free radical scavenging effect. These enzymes can regulate the oxidation-reduction states of cysteines transcription factors thereby modulating their activity. (Allen, J.F. (1993) FEBS Lett. 332, 203-207; Storz, G., et al., (1990) Science 248, 189-194; Toledano, M.B., et al., (1994) Cell 78, 897-909; Galang, C.K. et al., (1993) Mol. Cell. Biol. 13, 4609-4617; Pognonec, P., et al., (1992) J. Biol. Chem. 267, 24563-24567; Rigoni, P., et al., (1993) Biochim. Biophys. Acta 1173, 141-146; Gehring, W.J. (1994) Annu. Rev. Biochem 63; 478-526).

TABLE VI

Effect of Sox-4 on the Activity of class I-CAT chimeras or TG-CAT chimeras when cotransfected into FRTL-5 thyroid cells maintained in 5H medium plus 5% serum

Class I CAT Chimera	CONTROL Conversion Rate (% pSVO Control)	+ Sox-4 Conversion Rate (% pSVO Control)	RATIO (+ Sox-4/ no Sox-4)	TG-CAT Chimera	RATIO (+ Sox-4/ no Sox-4)
pSVO	1.0	1.0 ±0.2	1.0	P(-828)	***2.0***
p(-1100)	5.0 ±1	2.5 ±0.8	**0.5**	p(-688)	***2.7***
p(-400)	22.5 ±2	10.2 ±1.3	**0.45**	p(-206)	1.0
p(-203)	28.4 ±2.9	10 ±0.9	**0.35**	p(-170)**	1.2
p(-168)	23 ± 1.8	6.2 ± 1.1	**0.27**		
p(-127)	4.1 ± 1.2	3.2 ± 0.9	0.78		
p(-127NP) CRE	8.9 ± 1.3	3.5 ± 0.8	**0.39**		
p(-68) CRE	3.6 ±1.0	3.5 ±1.0	0.97		

Bold values indicate significant suppression by Sox-4 ($P<0.05$ or better).
Bold and italicized values represent significant enhancement.
**This clone contains oligo K site which is the insulin response element of the TG promoter (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317; Francis-Lang, H., et al., (1992) Mol. Cell Biol. 12, 576-588; Aza Blanc, P., et al., (1993) Mol. Endocrinol. 7, 1297-1306).

Figure Legend for Table VI. FRTL-5 cells were grown to near confluency in 6H medium (plus TSH) then were maintained in 5H medium (no TSH) for 7 days before being transfected with the noted chimeras, as described in Examples 7 and 8 and Figure 16A, using DEAE Dextran. Control cells were transfected with vector alone. CAT activity was measured as described (Example 7; Example 8, Materials and Methods). The absence of an effect on p(-127), but a significant effect on p(-127NP)CRE, is consistent with the data in Figure 19C vs 19B.

TABLE VII

Effect of MMI on the activity of class I-CAT chimeras or on thioredoxin activity when Sox-4 is cotransfected into FRTL-5 thyroid cells maintained in 5H medium plus 5% serum.

Class I CAT Chimera	CHIMERA ACTIVITY NO MMI RATIO (+ Sox-4/ no Sox-4)	CHIMERA ACTIVITY PLUS MMI RATIO (+ Sox-4/ no Sox-4)	THIOREDOXIN ACTIVITY PLUS Sox-4 NO MMI (% of CONTROL)	THIOREDOXIN ACTIVITY PLUS MMI (% of CONTROL)
pSVO	1.0	1.0 ±0.2	1.0	*2.6*
p(-1100)	0.5	**0.15**	**1.0**	*2.9*
p(-400)	0.45	**0.2**	**1.0**	*3.2*

Bold values indicate significant suppression by Sox-4 plus MMI more than Sox-4 without MMI ($P<0.05$).
Bold and underlined values indicate no significant effect by transfection.
Bold and italicized values indicate significant effect by MMI.

Figure Legend for Table VII. FRTL-5 cells grown to near confluency in 6H medium (plus TSH) then maintained in 5H medium (no TSH) for 7 days before being transfected with a control plasmid or a plasmid containing Sox-4 DNA. Cells were then treated with or without 5mM MMI for 40 hours. CAT activity was measured as described (Example 7; Example 8, Materials and Methods). The MMI treatment decreased CAT activity of Sox-4 transfectants further than in cells transfected with control vector ($P<0.05$). Figure 16B notes the structure of each chimeric CAT construct used.

Example 9

Identification of a Downstream Silencer and its Role in Hormone and MMI Reduction of Class I Levels

Materials and Methods

Materials. TSH, hormones, and other materials are the same as in Examples 6 and 8.

Cell culture. FRTL-5 rat thyroid cells (Interthyr Research Foundation, Baltimore, MD; ATCC No. CRL 8305) were a fresh subclone (F1) that had all properties previously detailed (Saji, M., et al. (1992a); Saji, M., et al. (1992b); Kohn, L.D., et al. (1992) Intern. Rev. Immunol. 9, 135-165; Kohn, L.D., et al. (1995) In: Vitamins and Hormones (Litwack, G., ed.) Academic Press, San Diego 50, 287-384; Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 793-804; Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al. (1995) Endocrinology, 136, 269-282; Shimura, H., et al. (1995) Mol. Endocrinol. 9, 527-539; Shimura, Y., et al. (1994) J. Biol. Chem. 269, 31908-31914; Kohn, L.D., et al. (1986) Sept. 2, U.S. Patent 4,609,622) (Examples 6 and 8). In different experiments, as noted, cells were maintained in 5h medium which contains no TSH or 3H medium which contains no TSH, insulin, or hydrocortisone.

Construction of MHC class I promoter-CAT chimeric plasmids. Construction of the CAT chimeras of the PD1 swine 5'-flanking sequences, p(-1100)CAT, p(-549)CAT, p(-400)CAT, p(-203)CAT, and p(-127)CAT has been described (Examples 6, 7, 8; Ehrlich, R., et al. (1988) Mol. Cell Biol. 8, 695-703; Weissman, J.D. and Singer, D.S. (1991) Mol. Cell. Biol. 11, 4217-4227; Maguire, J.E., et al. (1992) Mol. Cell. Biol. 12, 3078-3086; Howcroft, T.K., et al. (1993) EMBO J. 12, 3163-3169; Giuliani, C., et al. (1994) J. Biol. Chem. 270:11453-11462) (Examples 7 and 8).

Plasmid pSVGH, constructed to evaluate

transfection efficiency (Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 1701-1715), was a BamHI-EcoRI fragment encoding the human growth hormone (hGH) gene, isolated from p0GH (Nichols Institute, San Juan Capistrano, CA) and inserted into the BamHI-XbaI site of the pSG5 expression vector (Stratagene, La Jolla, CA).

Transient expression analysis - Transient transfections using FRTL-5 cells have been described previously (Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al. (1995) Endocrinology, 136, 269-282; Shimura, H., et al. (1995) Mol. Endocrinol. 9, 527-539; Shimura, Y., et al. (1994) J. Biol. Chem. 269, 31908-31914; Giuliani, C., et al. (1995) J. Biol. Chem. 270:11453-11462. Examples 7 and 8).

CAT assays were performed as described (Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al. (1995) Endocrinology, 136, 269-282; Shimura, H., et al. (1995) Mol. Endocrinol. 9, 527-539; Shimura, Y., et al. (1994) J. Biol. Chem. 269, 31908-31914; Giuliani, C., et al. (1994) J. Biol. Chem. 270:11453-11462. Gorman, C.M., et al. (1982) Mol. Cell Biol. 2, 1044-1051, Examples 7 and 8) using 10-30 μg cell lysate in a final volume of 130 μl. Incubation was at 37° for 4 hours with acetylCoA supplementation (20 μl of a 3.5 mg/ml solution) after 2 h.

Cellular extracts. Cell extracts were made by a modification of the method of Dignam et al. (Dignam, J., et al. (1983) Nucleic Acids Res. 11, 1475-1489; Examples 6 and 8). Nuclear extracts also used were made with the procedure in Example 8.

Electrophoretic mobility shift assays (EMSA) - Oligonucleotides used for EMSA were synthesized or were purified from 2 % agarose gel using QIEAX (Quiagen, Chatsworth, CA) following restriction enzyme treatment of

the chimeric CAT constructs described above (Examples 6 and 8).

Electrophoretic mobility shift assays were performed basically as previously described (Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al. (1995) Endocrinology, 136, 269-282; Shimura, H., et al. (1995) Mol. Endocrinol. 9, 527-539; Shimura, Y., et al. (1994) J. Biol. Chem. 269, 31908-31914; Giuliani, C., et al. (1995) J. Biol. Chem. 270:11453-11462; Hennighausen, L. and Lubon, H. (1987) Methods Enzymol. 152, 721-735, Examples 6, 8, 10 and 11).

In experiments using CREB and other antiserum, extracts were incubated in the same buffer containing antiserum or normal rabbit serum at 20° for 1 h before being processed as above.

Footprinting Using the 1,10-Phenanthroline-Copper Ion Procedure. Footprinting, using 1,10-phenanthroline-copper ion, was carried out essentially as described by Kuwabara and Sigman (Kuwabara, M. D. and Sigman, D. S. (1987) Biochemistry 26, 7234-7238). After scaling-up the EMSA using an end-labelled fragment, Fr168, comprising -168 through -1 bp of the PD1 promoter, the gel was immersed in 200 ml of 50 mM Tris-HCl, pH 8.0 and 20 ml each of the following solutions were added: 2 mM 1,10-orthophenanthroline containing 0.45 mM $CuSO_4$ and 58 mM 3-mercaptopropionic acid. After 15 min at room temperature, 20 ml of 28 mM 2,9-dimethylorthophenanthroline was added to quench the reaction; and, after 2 min, the gel was rinsed extensively in distilled H_2O and autoradiographed for 40 min at 4°C until the retarded bands were visible. Bands with protein/DNA complexes of interest were excised and eluted overnight at 37°C in 0.5 M ammonium acetate containing 0.1% sodium dodecyl sulfate (SDS) and 10 mM magnesium acetate. The eluted DNA with was precipitated with

ethanol and resuspended in distilled H_2O. Equal numbers of counts of each sample were dried, resuspended in 98 % formamide containing 10 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol, and separated on an 8 % sequencing gel along with G+A and C+T Maxam-Gilbert sequence reactions (Maxam, A. M. and Gilbert, W. (1980) Methods Enzymol. 65, 499-560) performed using the same probe. Autoradiography was at -80 C overnight.

RESULTS

The CRE-like Sequence Between -107 and -100 bp Functions as a Constitutive Silencer Element. MHC class I gene transcription in thyroid cells is repressed by TSH, through its cAMP signal (Saji, M., et al. (1992a) Saji, M., et al. (1992b). One of these studies (Saji, M., et al. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1944-1948) mapped the TSH response to within 127 bp of initiation of transcription. Examination of the sequence in this DNA segment (Figure 9) revealed the presence of an 8 bp sequence (-107 to -100 bp) with homology to characterized CREs (Montminy, M.R., et al. H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6682-6686; Habener, J.F. (1990) Mol. Endocrinol. 4, 1087-1094). To determine whether this CRE-like element functions to regulate class I promoter activity, a set of derivative constructs was generated from a parental construct containing 127 bp of 5' flanking sequence p(-127CAT). In one derivative, the 8 bp CRE-like sequence was simply deleted; in the other, a nonpalindromic mutation of the CRE octamer was substituted for the CRE-like sequence. Both constructs displayed increased promoter activity, relative to the parental construct, when transfected into FRTL-5 cells maintained in 3H medium plus 5% calf serum (Fig. 25A) or 5H medium plus 5% calf serum (data not shown).

The ability of the CRE-like element to silence a heterologous promoter was assessed by introducing a 38 bp DNA segment, spanning -127 to -90 bp, downstream of an

SV40 minimal promoter (Ibuyama S. et al. (1992) Mal. Endocrinol., 6:1701-1715; Shimura H., et al, (1994) Mol. Endocrinol, 8:1049-1069; Ohmori M. et al. (1995) Endocrinology, 136:269-282; Shimura Y., et al. (1994) J. Biol. Chem., 269:31909-31914) (Fig. 25B). When placed in a 5' to 3' orientation, a single copy of this DNA segment was able to significantly reduce SV40 promoter activity; and the magnitude of the effect increased with the number of copies of the 38 bp segment inserted (Fig. 25B). When placed in a 3' to 5' orientation, two copies of this DNA segment were able to significantly reduce SV40 promoter activity. Derivatives of the 38 bp segment, containing either a deletion or nonpalindromic mutation of the CRE-like element, had no significant effect on SV40 promoter activity (Fig. 25B). These data (Fig. 25B) were obtained in cells maintained in 3H medium plus 5% calf serum; the same results were obtained with cells maintained in 5H medium plus 5% calf serum (data not shown).

From these data, it was concluded that the 8 bp CRE-like site is important in FRTL-5 rat thyroid cells for the function of a constitutive silencer located in a 38 bp fragment of the class I 5'-flanking region, -127 to -90 bp from the start of transcription. In addition, we conclude that expression of the silencer activity related to the CRE-like site is unaffected by the presence or absence of hydrocortisone; we have separately (Giuliani, C., et al. (1995) J. Biol. Chem. 270:11453-11462) located the hydrocortisone action to suppress class I gene expression in FRTL-5 cells to a different element, Enhancer A, -180 to -170 bp from the start of transcription.

TSH or Forskolin Treatment of FRTL-5 Thyroid Cells Induces a Novel Protein/DNA Complex Whose Formation, Like Silencer Activity, Depends on the CRE-like Site. Because TSH/forskolin treatment of FRTL-5 cells results in reduced transcription from the class I promoter (Saji, M., et al. (1992) Proc. Natl. Acad. Sci. U. S. A. 89,

1944-1948; Saji, M., et al. (1992) J. Clin. Endocrinol. Metab. 75, 871-878), it was of interest to determine whether TSH/forskolin altered or induced the formation of any novel protein/DNA complexes with the region containing the CRE-dependent silencer activity. A DNA fragment extending from -168 bp to +1 bp (Fr168; Fig. 26A) was used in gel mobility shift assays with extracts derived from FRTL-5 cells cultured with or without $1x10^{-10}$ M TSH for 48 hours (Fig. 26B). A multiplicity of protein/DNA complexes were formed with either extract. The protein/DNA complexes A to D were common to both extracts, were not altered by TSH treatment of the cells, and appeared to derive from protein interactions with DNA sequences located between -89 and +1. This was suggested by the fact that 100-fold excess of unlabeled fragment 127 (Fr127), -127 to +1 bp, could compete for these complexes, whereas the CRE-like silencer element extending from -127 (Figure 26B, lanes 6 and 7) to -90 bp, termed CRE-1, had no effect on these protein/DNA complexes (Fig. 26B, lanes 4 and 8). The complex labeled E appears to be non-specific, since it is not eliminated by any competitor DNA fragment. It was notable, however, that TSH treatment of the FRTL-5 cells induced the formation of two novel complexes, F and G (Fig. 26B, lane 5 vs lane 1). As evidenced by the following, their formation was specific and required the CRE-like site important for silencer activity.

Formation of the TSH-induced F and G complexes could be prevented not only by unlabeled DNA fragments extending from -168 to +1 or from -127 to +1 bp (Fig. 26B, +TSH, lanes 6 and 7 vs 5), but also by the -127 to -90 bp fragment containing the CRE-like site, termed CRE-1 (Fig. 26B, +TSH, lane 8 vs 5). In addition, as illustrated in studies with the G complex, DNA footprint analyses of the TSH-induced complexes, using a procedure involving 1/10-phenanthroline-copper ions, identified a protected

region bounded by two strong hypersensitive sites, -131 to -95 bp. The CRE-like site, -107 to -100 bp, lies within this protected region and is demarcated by a less prominent hypersensitive band at -110 bp and the prominent site at -95 bp. A hypersensitive site at -103 bp in the middle of the CRE-like site was also observed. Similar data were obtained with complex F. These data established that the TSH-induced F and G complexes involved the 38 bp region which has silencer activity that is dependent on the CRE-like site. These data do not, however, restrict the downstream silencer region to this 38bp segment; the silencer activity of this segment can extend to 10 nucleotides in the 3' direction to -80 bp of the PDI class I 5' flanking region. Thus a 48 bp segment -127 to -80 bp, duplicates all activity of the 38 bp silencer in this and subsequent experiments and its activity is CRE dependent.

Forskolin (10 μM) could substitute for TSH to induce the formation of the F and G complexes and the formation of both was prevented by the -127 to -90 bp CRE-1 DNA fragment with the CRE-like site. Moreover, using extracts from forskolin- as well as TSH-treated cells (data not shown), we showed that their formation was also prevented by the -127 to -90 bp fragment, wherein a consensus CRE sequence was substituted for the CRE-like sequence, but not by derivative oligonucleotides from which the CRE-like element had been removed, either by deletion (ΔCRE) or a nonpalindromic (NP-CRE) substitution. The region of the 38 bp silencer 5' to the CRE was not able to inhibit formation of the TSH/cAMP-induced complex nor was a shortened form of CRE-1, termed CRE-2, with only 6 base pairs on either side of the CRE octamer. These data established that the TSH-induced formation of the novel F and G complexes is mediated by the cAMP signal of TSH, exactly as is TSH-induced suppression of class I RNA levels (Saji, M., et al. (1992) Proc. Natl. Acad. Sci. U.

S. A. 89, 1944-1948; Saji, M., et al. (1992) J. Clin. Endocrinol. Metab. 75, 871-878), and that formation of the TSH/cAMP-induced complexes requires the CRE-like sequence important for silencer activity. They additionally suggest that sequences flanking the CRE-like site might be involved in complex formation, consistent with the extended DNA footprint and studies of complexes with other CRE sites (Montminy, M.R., et al. H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6682-6686; Habener, J.F. (1990) Mol. Endocrinol. 4, 1087-1094; Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 1701-1715).

Similar EMSA data were obtained using radiolabeled Fr127, -127 to +1 bp, with extracts derived from FRTL-5 cells incubated with or without TSH. As with Fr168, extracts from either TSH-treated or untreated cells generated a series of complexes, all of which could be competed by unlabeled Fr127 or Fr168. Once again, TSH or forskolin induced the appearance of a novel protein/DNA complex. The TSH-induced complex could be specifically competed by unlabeled CRE-1, the 38 bp DNA fragment extending from -127 to -90 bp. In contrast, derivative oligonucleotides from which the CRE-like element had been removed, either by deletion (ΔCRE) or substitution (NP CRE), were unable to compete for or form the TSH-induced band. A derivative into which a consensus canonical CRE-site was introduced (CON-CRE) was as efficient in competition as the native sequence (CRE-1). A DNA fragment containing only sequences 5′ of the CRE-like element, from -127 to -108 bp, could not compete for the TSH-induced band.

MMI and TSH Induce the Formation of a Protein Complex with the CRE-like Sequence of the 38 bp Downstream Silencer, -127 to -89 bp; Its Formation and Function Depends on a Protein Interacting With the Insulin Response Element of the TSH Receptor (TSHR) In the above results (Figure 26), we used electrophoretic mobility shift assays

(EMSA) and radiolabeled DNA fragments extending from -168 or -127 bp to +1 bp to identify a TSH/cAMP-increased protein/DNA complex interacting with the CRE site of the 38 bp silencer. Treatment of the FRTL-5 cells with 5 mM MMI (Fig. 27A, lane 2 vs 1, arrow), as well as TSH (Fig. 27A, lane 5 vs 1, arrow), induced the formation of a similarly sized protein DNA complex with the radiolabeled 168 bp fragment. Treatment with TSH plus MMI increased formation of the protein DNA/complex more than either treatment alone (Fig. 27A, lane 4 vs 2 or 5); in 7 experiments, when increases were quantitated densitometrically, the ratio of the complexes induced by TSH, MMI, or both was, respectively, 1 ± 0.2, 0.8 ± 0.3, and 2.2 ± 0.3.

The ability of FRTL-5 cell extracts to form the TSH-induced complex with the radiolabeled 168 bp fragment is prevented by an unlabeled oligonucleotide with the 38 bp sequence of the silencer element, -127 bp to -90 bp (Figure 26B), but not by an oligonucleotide with the CRE-like site within the silencer deleted or with the CRE-like element replaced by a nonpalindromic mutation. The complex induced by MMI is also prevented by a 250-fold excess of the unlabeled 38 bp silencer, termed CRE-1 (Fig. 27B, lane 3 vs 2) but not by the same amount of silencer oligonucleotide with a nonpalindromic substitution of the CRE-like site (Fig. 27B, lane 4 vs 2). Thus, formation of the MMI- as well as the TSH-increased silencer complex requires an intact CRE-like sequence.

Formation of the protein/DNA complex induced by MMI, TSH, or both involve an insulin/serum-sensitive factor. Thus, a 200-fold excess of an oligonucleotide having the sequence of the insulin-responsive element (IRE) of the TSHR, oligo TIF (TSH receptor insulin-response factor) (Shimura, Y., et al., (1994) J. Biol. Chem. 269, 31908-31914), was able to prevent the formation of the complex induced by MMI (Fig. 27A, lane 3

vs 2), TSH (Fig. 27A, lane 9 vs 5) and MMI plus TSH (Fig. 27A, lane 8 vs 4) when added to the binding mixture in vitro. The effect of oligo TIF was specific, since an oligonucleotide having the sequence of the insulin response element of the TG promoter, oligo K (Santisteban, P., et al., (1992) Mol. Endocrinol. 6, 1310-1317), did not prevent the formation of the complex induced by TSH (Fig. 27A, lane 7 vs 5 and 9) or by MMI plus TSH (Fig. 27A, lane 6 vs 4 and 8). The lack of involvement of Sox-4, the protein reactive with oligo K, in the TSH-induced or MMI-induced complex is consistent with the dominance of the downstream CRE-containing silencer. However, if the CRE is deleted or mutated, Sox-4 can act downstream as evidenced in Figure 19C by the ability of oligo K to increase CAT activity of p(-127NP) CAT. This reflects the presence of two Sox-4 reactive sites, one between the interferon response element of approximately -161bp and the downstream silencer at -127bp, the other between -89 and -68bp. These are expressed only when the CRE, -107 to -100bp, is mutated or deleted (Figure 19C and Table VI).

The same data were evident using the -127 to +1 bp fragment. Thus, 5 mM MMI treatment of the cells induced the formation of a complex when fragment 127 was substituted as the radiolabeled probe. The complex had the same mobility as the TSH-induced complex. MMI plus TSH additively increased the formation of the complex; and oligo TIF added in vitro, but not oligo K, prevented MMI/TSH-induced complex formation. Formation of the TSH/MMI-induced complex was prevented by including a 250-fold excess of the unlabeled 38 bp silencer, CRE-1, in the incubation. Finally, MMI-treated extracts did not form the new complex when the radiolabeled 127 bp fragment had a nonpalindromic substitution of the CRE-like site, indicating the CRE-like octamer is necessary to form the MMI-induced complex.

The TSHR insulin response element, -220 to -188 bp, has been found to have two regions able to form protein/DNA complexes with FRTL-5 cell extracts (Shimura, Y., et al., (1994) J. Biol. Chem. 269, 31908-31914). Proteins interacting with the more 3' region (Fig. 28C, black bar) are associated with insulin responsiveness, since a mutant of this region, mutant 1 (Fig. 28C), loses insulin responsiveness after CAT chimeras of the minimal TSHR promoter are transfected into FRTL-5 cells. Mutant 1, also looses Y-box protein reactivity (Example 11). Proteins interacting with the 5' region (Fig. 28C, cross hatched area) are single strand binding proteins; mutation of this region, as in mutant 2 (Fig. 28C), retain insulin responsiveness but lose their ability to bind the single strand binding proteins (Shimura, Y., et al., (1994) J. Biol. Chem. 269, 31908-31914). The swine class I promoter-CAT chimera was transfected into FRTL-5 cells with a plasmid containing an oligonucleotide with the sequence of one or the other oligo TIF mutants or oligo K and half of each set of transfected cells was treated with MMI. Cotransfection of the TIF mutant 2 oligonucleotide with p(-127)CAT resulted in a significant ($P<0.01$) loss in MMI-decreased promoter activity (Fig. 28B) but no significant change in basal or constitutive activity. There was no effect on MMI activity in cells cotransfected with the plasmid containing oligo K, the TG insulin response element, as will be evident below. It was also not duplicated by cotransfection with the plasmid containing oligo TIF mutant 1 (data not shown). Cotransfection of the plasmid containing oligo TIF mutant 2 eliminated the MMI-induced decrease evident in the p(-1100)CAT chimera (Fig. 28A), suggesting its effect on the downstream silencer is a dominant effect on MMI action to decrease promoter activity. The ability of oligo TIF to increase the basal activity of p(-1100)CAT without MMI (Fig. 28A) may reflect the interactive relationship the

downstream silencer and the enhancer associated with the upstream silencer, which will be described below. (Example 10)

When the oligo TIF mutant 2 is transfected into cells, it binds the insulin responsive factor important in the formation of the MMI/TSH-induced protein DNA complex with the downstream silencer. It thereby prevents the MMI/TSH-induced decrease in class I promoter activity. The inability of oligo K or oligo TIF mutant 1 to prevent the MMI-induced decrease in promoter activity insures that the effect is specific for the factor interacting with the TSHR insulin response element and that it is the insulin-sensitive factor and/or the Y-box protein, TSEP-1, rather than the single strand binding protein (SSBP) which can interact with the oligo TIF sequence (See Example 11). These data support the conclusion that MMI and TSH independently and additively induce the formation of a protein complex with a 38 bp silencer element between -127 and -89 bp and that formation of the complex in each case requires the presence of the CRE-like site within the silencer. Its formation requires, in addition, an insulin/serum-responsive factor which also interacts with the insulin response element in the TSHR minimal promoter. Formation of the MMI/TSH-increased complex with the silencer (Figs. 26 and 27) is functionally associated with the MMI/TSH effect on class I gene expression (Fig. 28).

The 38 bp Class I Region Containing the CRE-dependent Silencer Element Forms Complexes with Multiple Proteins, Some of Which are Involved in cAMP-induced Negative Regulation of TSHR. To characterize proteins capable of interacting with the 38 bp silencer element, a double-stranded oligonucleotide spanning the segment -127 to -90 bp was radiolabeled and used in gel shift assays with FRTL-5 cell extracts (Fig. 29). Four sets of complexes were observed (A-D), all of which appeared to be specific, since their formation was

prevented by competition with unlabeled probe (Fig. 29A, lanes 3-6 vs 2), albeit with different affinities. Like the TSH/MMI-induced complex with Fr168 (Figs. 26 and 27) or Fr127, formation of all the complexes was dependent on the CRE-like element. Thus, their formation was not inhibited by the 38 bp silencer in which the CRE was deleted (Fig. 29A, ΔCRE-1, lanes 7-9, vs 2) or mutated to its nonpalindromic form.

One of the protein/DNA complexes formed with the 38 bp silencer in the A region was inhibited by an oligonucleotide, 5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3' from Promega, which contains a consensus CRE (underlined) but 9-10 otherwise unrelated flanking nucleotides from the somatostatin CRE (Fig. 29A, lanes 10-12 vs 2). The same complex could be super-shifted (Fig. 29B, lane 4) with antibody to CRE binding protein-327 (CREB) (Waeber, G., et al. (1991) Mol. Endocrinol. 5, 1418-1430) but not (Fig. 29B, lanes 1-3) by anti-CREB2, anti-mXBP, or anti-activating transcription factor-2 (ATF2-BR). One protein interacting with the CRE-like site in the 38 bp silencer, -127 to -90 bp can, therefore, be identified as CREB (Montminy, M.R., et al. H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6682-6686; Habener, J.F. (1990) Mol. Endocrinol. 4, 1087-1094; Waeber, G., et al. (1991) Mol. Endocrinol. 5, 1418-1430; Hoeffler, J.P., et al. (1988) Science 242, 1430-1433; Deutch, P.J., et al. (1988) J. Biol. Chem. 263, 18466-18472) or an immunologically related CRE binding protein.

Two of the double strand binding proteins in the A and B complexes in Figure 29A, in addition to CREB, are thyroid transcription factor-1 (TTF-1) and Pax-8. TTF-1 is a thyroid-specific transcription factor important for full expression of the TSHR in FRTL-5 thyroid cells (Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al. (1995) Endocrinology, 136, 269-282; Shimura, H., et al. (1995) Mol. Endocrinol. 9, 527-539;

Civitareale, D., et al. (1993) Mol. Endocrinol. 7, 1589-1595). TTF-1 and Pax-8 are necessary for thyroid-specific expression of the thyroglobulin and thyroid peroxidase genes (Civitareale, D., et al. (1989) EMBO J. 8, 2537-2542; Guazzi, S., et al. (1990) EMBO J. 9, 631-3639; Francis-Lang H, et al. (1992) Mol Cell Biol 12:576-588; Kikkawa, F., et al. (1990) Mol. Cell. Biol. 10, 6216-6224; Mizuno, K., et al. (1991) Mol. Cell. Biol. 11, 4927-4933; Lazzaro, D., et al. (1991) Development 113, 1093-1104; Zannini, M., et al. (1992) Mol. Cell. Biol. 12, 4230-4241). TTF-1 is a homeodomain-containing, DNA-binding protein which is expressed from the onset of thyroid differentiation (Civitareale, D., et al. (1989) EMBO J. 8, 2537-2542; Guazzi, S., et al. (1990) EMBO J. 9, 631-3639; Francis-Lang H, et al. (1992) Mol. Cell. Biol. 12:576-588; Lazzaro, D., et al. (1991) Development 113, 1093-1104). Pax-8 is a paired domain-containing protein which binds to a sequence overlapping one of the TTF-1 recognition sites in the thyroglobulin and thyroid peroxidase genes and is also involved in thyroid differentiation (Zannini, M., et al. (1992) Mol. Cell. Biol. 12, 4230-4241). The B complex in Figure 29A formed with the 38 bp class I silencer comprises a protein/DNA adduct with TTF-1; the A complex in Figure 29A involves a Pax-8 adduct in addition to CREB. This is evidenced as follows.

Formation of the B complex in Figure 29A with the 38 bp class I silencer is inhibited by an oligonucleotide with the sequence of the TTF-1 binding element from the TSHR [Fig. 30B, lane 5 (TSHR oligo TTF-1) vs 2], but not by a mutant form of the oligonucleotide which loses its reactivity with TTF-1 (Fig. 30B, lane 6 vs 2). The TSHR TTF-1 binding site does not interact with Pax-8 (Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al. (1995) Endocrinology, 136, 269-282; Shimura, H., et al. (1995) Mol. Endocrinol. 9,

527-539; Civitareale, D., et al. (1993) Mol. Endocrinol. 7, 1589-1595). The same data were obtained in the presence of 3 (Fig. 30B) or 0.5 μg poly dI-dC. The sequences of each oligonucleotide are noted in Figure 30C; their characteristics, their specificity for TTF-1, and their inability to bind Pax-8 have been separately detailed (Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al. (1995) Endocrinology, 136, 269-282; Shimura, H., et al. (1995) Mol. Endocrinol. 9, 527-539; Shimura, Y., et al. (1994) J. Biol. Chem. 269, 31908-31914).

The oligonucleotide which mimics the site on the thyroglobulin promoter which interacts with TTF-1 or Pax-8 is termed oligo C (Civitareale, D., et al. (1989) EMBO J. 8, 2537-2542; Guazzi, S., et al. (1990) EMBO J. 9, 631-3639; Francis-Lang H, et al. (1992) Mol. Cell. Biol. 12:576-588). The sequences of oligo C and a mutant of oligo C which has been shown to no longer act with TTF-1 or Pax-8 (Civitareale, D., et al. (1989) EMBO J. 8, 2537-2542; Guazzi, S., et al. (1990) EMBO J. 9, 631-3639; Francis-Lang H, et al. (1992) Mol. Cell. Biol. 12:576-588) are noted in Figure 30C. As would be expected, since it interacts with TTF-1, oligo C can prevent formation of the B complex in Figure 29A in either 3 or 0.5 μg poly dI-dC [Fig. 30B or 30A, lane 4 (TG oligo C) vs 2]. The oligo C mutant (TG oligo C Mut.) does not, in contrast, prevent formation of the B complex (Fig. 30A, lane 5 vs 2; Fig. 8B, lane 3 vs 2). This is consistent with the data above which shows that complex B involves TTF-1, as evidenced with the TSHR oligonucleotide which is specific for TTF-1. Of interest, however, a portion of the A complex in Figure 29A is inhibited by oligo C (Figs. 30A or 30B, lane 4 vs 2), but not by its mutant (Fig. 30A, lane 5 vs 2; Fig. 30B, lane 3 vs 2), nor by the TSHR oligonucleotide reactive only with TTF-1 (Fig. 30B, lane 5 vs 4 or 2). This indicates that a portion of the A complex in Figure

29A represents a CRE-dependent interaction between Pax-8 and the 38 bp silencer, in addition to CREB.

Two other proteins interacting with the 38 bp CRE-1 oligonucleotide in a CRE-dependent manner are (a) a single strand binding protein (SSBP) which binds to the noncoding strand of the TSHR promoter, immediately 5' and contiguous with the TTF-1 site (Shimura, H., et al. (1995) Mol. Endocrinol. 9, 527-539) and (b) a Y-box protein, TSEP-1 (TSHR suppressor element protein-1), which binds to the coding strand of the TSHR at site identified by the existence of a decanucleotide tandem repeat, -163 to -141 bp in the minimal TSHR promoter (Example 11). This was evidenced when the following possibilities were evaluated: (a) that the decreased appearance of the C complex (Figure 29A) in the presence of higher concentrations of poly(dI-dC) (Figure 30B) in the binding assays reflected a less stringent sequence-specific binding reaction exhibited by single-strand binding proteins and (b) that both SSBP and TSEP-1 were involved.

First, formation of the C, but not the A and B complexes of Figure 29A, with the double-stranded 38 bp silencer termed CRE-1 was decreased by including either the coding or noncoding strand of CRE-1 as an unlabeled competitor in the binding reaction (Figure 31, lanes 2 and 3 vs 1, respectively). This suggested that the C complexes involved the binding of proteins which could also bind single strand DNA. Second, a single stranded oligonucleotide including the SSBP binding site on the TSHR noncoding strand, -194 to -169 bp (Figure 31C) and one including the TSEP-1 binding site, -177 to -138 bp, on the coding strand of the TSHR (Fig. 9C), each inhibited the formation of the C complexes with the double-stranded 38 bp class I CRE-1 silencer element, -127 to -90 bp (Figure 31B, lanes 3, 4 and 6, 7, vs 2 and 5, respectively). These data suggested that the single strand binding proteins interacting with the 38 bp

silencer in a CRE-dependent manner included two proteins important for TSH/cAMP-induced suppression of the TSHR, in addition to TTF-1.

Third, in competition studies using the coding and noncoding strands of the 38 bp CRE-1 silencer as radiolabeled probes (Fig. 32), TSEP-1, a Y-box protein which interacts with three sites on the TSHR (Figure 32A, bottom), inhibits formation of a specific protein/DNA complex with the strand coding of CRE-1 (Figure 32A). Each TSHR Y-box binding site contains a CCTC motif (Example 11). Mutations of this motif (Mut. 2) result in a loss or decrease in TSEP-1 binding to the TSHR and decreased TSEP-1 suppression activity by comparison to wild type sequence or another mutation (Mut. 1) not involving the CCTC motif (Figure 32A, bottom). Using the CRE-1 coding strand as radiolabeled probe, formation of a major protein complex at the top of the gel was prevented or reduced by including an excess of wild type or Mut. 1 oligonucleotide (Figure 32A, lane 2 vs lanes 3-4, 6-7, and 9-10, respectively) but not Mut. 2 oligonucleotide (Figure 32A, lane 2 vs lanes 5, 8, and 11, respectively). The SSBP binding domain on the TSHR (Figure 32B, bottom, dark bar) is 5' and contiguous with the TTF-1 binding domain on the noncoding strand of the TSHR; mutation of two G nucleotides (Figure 32B, bottom, underlined) results in the decreased SSBP binding to the single strand oligonucleotide containing the TSHR SSBP site but not decreased TTF-1 binding to the double strand oligonucleotide with the same mutation (Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al. (1995) Endocrinology, 136, 269-282). Using the CRE-1 noncoding strand as radiolabeled probe, formation of a major protein complex was reduced by including an excess of wild type, single strand oligo able to bind SSBP (Figure 32B, lane 2 vs lanes 3 and 5) but much less so by

the mutated SSBP oligonucleotide (Figure 32B, lane 2 vs lanes 4 and 6).

The 38 bp CRE-1 region of the class I 5'-flanking region, -127 to -90 bp, which exhibits CRE-dependent silencer activity and which is involved in the formation of novel TSH/cAMP-induced protein complexes that are also CRE-dependent, interacts with a multiplicity of proteins in a CRE-dependent manner. Five of these proteins can be identified herein, CREB, TTF-1, Pax-8, TSEP-1, and SSBP. Four interact with the TSHR minimal promoter in FRTL-5 thyrocytes: CREB, TTF-1, TSEP-1, and SSBP. Three are known to be important for TSH/cAMP-induced negative regulation of the TSHR in FRTL-5 thyroid cells: TTF-1, SSBP, and TSEP-1 (Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 793-804; Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al. (1995) Endocrinology, 136, 269-282; Shimura, H., et al. (1995) Mol. Endocrinol. 9, 527-539; Shimura, Y., et al. (1994) J. Biol. Chem. 269, 31908-31914; Example 11). One, TSEP-1, is a Y-box protein. A human Y-box protein, YB-1, also interacts with the MHC class II promoter and is important for TSH/cAMP-induced suppression of class II genes in lymphocytes (Ivashkiv, L.B. et al. (1991) J. Exp. Med. 174, 1583-1592; Vilen, B.J., et al. (1992) J. Biol. Chem. 267, 23728-23734; Brown, A.M., et al. (1993) J. Biol. Chem. 268, 26328-26333; Ivashkiv, L.B., et al. (1994) Immunopharmacology 27, 67-77; Ting, J.P., et al. (1994) J. Exp. Med. 179, 1605-1611; Wright, K.L., et al. (1994) EMBO J. 13, 4042-4053; MacDonald, G.H., et al. (1995) J. Biol. Chem. 270, 3527-3533).

TSH Modulation of the Multiplicity of Proteins Interacting with the CRE-dependent 38 bp Class I Silencer Element. TSH/forskolin-treatment of FRTL-5 thyroid cells for 12 to 18 hours causes a maximal decrease in class I RNA levels (Saji, M., et al. (1992a) Proc. Natl. Acad.

Sci. U. S. A. 89, 1944-1948; Saji, M., et al. (1992b) J. Clin. Endocrinol. Metab. 75, 871-878). Extracts from cells treated with TSH for this period alter the amount and composition of the protein/DNA complexes formed with the 38 bp silencer region whose activity and binding depends on the CRE (Figure 33). Thus, TSH treatment results in markedly diminished formation of the A and B complexes in Figure 29A but increased formation of the C complexes (Figure 33, lane 4 vs lane 2). In the A complex, TSH significantly decreases the CREB interaction, as evidenced by a diminished ability of anti-CREB-327 to supershift the A complex. The TTF-1 B complex is also decreased significantly. The simultaneous decrease of both is of interest since the CRE binding proteins and homeodomain proteins are known to act synergistically in the TSHR (Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069) and somatostatin receptor (Leonard, J., et al. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6247-6251; Vallejo, M., et al. (1992) J. Biol. Chem. 267, 12868-12875; Leonard, J., et al. (1993) Mol. Endocrinol. 7, 1275-1283).

The addition of anti-CREB-327 in vitro mimics the TSH-treatment in vivo to similarly increase C complex formation (Fig. 33, lane 3 vs 2 by comparison to lane 4 vs 2) but does not, by comparison, change A or B complex formation. This shows the increase in C complex induced by TSH may reflect the decrease in TTF-1 and its synergism with CREB.

TSH treatment decreases SSBP interactions with the TSHR in parallel with decreased TTF-1 binding to the TSHR (Shimura, H., et al. (1995) Mol. Endocrinol. 9, 527-539); it does not decrease TSEP-1 binding to the TSHR (Example 11). This would suggest the apparent increase in C complex formation induced by TSH or anti-CREB-327 does not reflect an increase in the SSBP complex.

Since, therefore, TSEP-1 might be the protein which exhibited a relative increase in the CRE-dependent interaction with the 38 bp silencer, it was determined whether it was an important component of the TSH-induced increase in the novel complexes with FR168 or Fr127. An oligonucleotide was able to bind TSEP-1, but not one binding SSBP, TTF-1, CREB, or Pax-8 could decrease the formation of the TSH-increased complex with FR168 (Fig. 34A), thereby confirming this possibility. The TSEP-1 binding oligo used in this experiment is from the insulin-sensitive element of the minimal TSHR promoter, -220 to -188 bp (Figure 32B).

Since the CRE-like site has been shown to be a critical component of a constitutive silencer (This Example; Figures 25A-B)and to participate in the formation of TSH-induced complexes (This Example, Figures 26A-B, 27A-B), it was of interest to determine its role in negative regulation of class I gene expression by TSH/cAMP or MMI. FRTL-5 cells transiently transfected with the pCAT promoter constructs containing one two 38 bp DNA segments, spanning -127 to -90 bp, downstream of an SV40 minimal promoter. As noted earlier (Fig. 25B), when placed in a 5′ to 3′ orientation, a single copy of this DNA segment was able to significantly reduce SV40 promoter activity and the magnitude of the effect increased with the number of copies of the 38 bp segment inserted (Figure 35B, C). Forskolin treatment of transfected cells resulted in an additional decrease in promoter activity ($P<0.05$) when a single copy was present, but not if two copies were present (Figure 35B). The forskolin activity is duplicated by treating cells with $1x10^{-10}$M TSH or 5mM MMI (data not shown). This indicated that TSH/cAMP and MMI could increase the activity of the silencer.

When FRTL-5 cells were transiently transfected with the series of 5′ deletion constructs of the class I promoter ligated to CAT, all of which share a common 3′

terminus but differ in the length of upstream sequences (Figure 16A, Figure 35A), TSH was still able to decrease the promoter activity of p(-89)CAT (Figure 16A, 35A), from which the 38 bp silencer region containing the CRE, -107 to -100 bp is deleted. Therefore, despite the fact that the CRE-like element functions as a constitutive silencer, is required for the formation of TSH-induced protein/DNA complexes, and exhibits TSH/cAMP responsiveness in the pCAT promoter construct, other elements downstream of -89 bp are involved in cAMP repression of a class I promoter activity.

To test whether any DNA sequences within 89 bp of transcription initiation might be related to the 38 bp CRF-1 silencer and the formation of the TSH-induced protein/DNA complex by Fr168. The ability of the -89 to +1 bp fragment (Fr89) to compete for the TSH-induced complex formed by Fr168 was tested. As shown in Figure 36, lanes 3 and 4 vs lane 11, this 90 bp fragment could compete for the formation of the TSH-induced band as could FR127 (Figure 36, lane 2) or CRE-1 (Figure 36, lane 1). The precise residues in the 90 bp fragment that compete for complex formation have not yet been identified nor is it clear which factor exhibiting CRE dependent binding to the 38 bp silencer region binds to the 90 bp fragment.

TSEP-1 is a Y-box (Example 11), which binds to CRE-1 and is involved in the formation of the TSH-induced band with Fr168, as evidenced by competition with oligonucleotides binding TSEP-1 from the TSHR Y-box protein is known to interact at multiple sites of the MHC class I 5'-flanking region including sequence within -89 bp of the Class 1 promoter (Example 11).

Example 10

Coordinate Regulation of the Upstream Silencer/Enhancer and Downstream Silencer

As noted in Figure 19A-C, cotransfection of oligo K with the p(-127)CAT chimera had no effect on

activity. However, oligo K had a profound stimulatory effect on promoter activity when cotransfected with p(-127NP)CAT, the nonpalindromic CRE mutation of p(-127)CAT (Figure 19C). These data suggested that the protein interacting with oligo K (Sox-4, Figure 20) and important for upstream silencer function was able to interact downstream, but its interaction was only evident when the downstream silencer was inactivated. Removal of the insulin-induced protein which is reactive with the upstream silencer results in the expression of an enhancer activity normally negated in p(127)CAT chimera by the presence of the functional downstream silencer. There is, therefore, coordination between the upstream and downstream regions; the downstream silencer is, nevertheless, functionally dominant and suppresses an enhancer activity associated with the oligo K reactive protein. (Sox 4; Figure 20)

Coordination between the upstream and downstream promoter is also evidenced in EMSA. Thus, high concentrations of the unlabeled 140 Fragment prevented formation of the MMI or MMI/TSH-induced complex with the CRE of the downstream silencer, when the 168 bp construct was the radiolabeled probe (Figure 37A, lane 2 vs 1 and 3). One site on the 140 Fragment which interacts with proteins involved in the formation of the downstream silencer complex increased by TSH/MMI appears to be the enhancer element. Thus, oligo E9, which inhibits formation of only the enhancer complex of the 140 Fragment (Figure 17), inhibits formation of protein/DNA complexes formed by the downstream 38 bp silencer (Figure 37B, lane 8). Inhibition appears to be specific, since oligonucleotides with AP-1 or Oct-1 consensus sequences do not similarly inhibit complex formation with the downstream silencer (Figure 37B, lanes 6 and 7), nor does the Promega CRE oligonucleotide which represents the somatostatin CRE sequence (Figure 37B, lane 7). Moreover,

the oligonucleotide with the sequence of the unlabeled 38 bp silencer (Figure 37B, lane 3, CRE-1) prevents formation of all the complexes inhibited by E9.

There appears, therefore, to be an interaction involving proteins binding to the upstream enhancer and the downstream silencer, particularly those important in negative regulation of the downstream silencer and TSHR.

Example 11

TSEP-1, A Y-box Protein Is An Important Regulator Of MHC Class I, Its Activity Is TSH And MMI Regulated

Materials and Methods

Cloning of TSEP-1 - A λgt11 FRTL-5 thyroid cell cDNA expression library (Akamizu, T., et al. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5677-5681) was screened by Southwestern blotting (Vinson, C. R., et al. (1988) Genes Dev. 2, 801-806) using the ^{32}P-labeled coding strand oligonucleotide, ssTR2(+) (Shimura, H. et al. (1993) J. Biol. Chem. 268, 24125-24137.), which includes both decanucleotides of the tandem repeat (TR) sequence in the TSHR minimal promoter, -162 to -140 bp. The procedure was otherwise the same as for cloning Sox-4 in Example 8.

Cells - Buffalo rat liver cells (BRL 3A, ATCC CRL 1442) were grown in Coon's modified Ham's F-12 supplemented with 5 % fetal calf serum (Biofluids, Rockville, MD). FRTL-5 (ATCC CRL 8305) and FRT rat thyroid cells were in the same medium (Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 793-804; Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al. (1993) J. Biol. Chem. 268, 24125-24137; Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Shimura, Y., et al. (1994) J. Biol. Chem. 269, 31908-31914; Ambesi-Impiombato, F. S. (1986) Fast-growing thyroid cell strain. U.S. Patent 4, 608, 341; Ambesi-Impiombato, F. S. and Coon, H. G. (1979) Int. Rev. Cytol. (Suppl.) 10, 163-171; Examples 6, 7 and 8).

Protein production in E. coli - Recombinant protein was produced using the pET system (Novagen, Madison, WI). TSEP-1 cDNA insert was ligated to the EcoRI site of the expression vector, pET-30(+), allowing the His-Tag sequence to be linked to its N-terminus. After transformation using E. coli BL21 (DE3), the procedure described in Example 8 for Sox-4 was followed.

EMSA - Assays used synthetic single- or double-stranded oligonucleotides, end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, then purified on 8 % native polyacrylamide gels (Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al. (1993) J. Biol. Chem. 268, 24125-24137; Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Shimura, Y., et al. (1994) J. Biol. Chem. 269, 31908-31914); Examples 6 and 8). One μg FRTL-5 nuclear extract or 50 ng recombinant TSEP-1 were incubated with or without unlabeled competitor oligonucleotides as described in Examples 6 and 8. DNA-protein complexes were separated on 5 % native polyacrylamide gels.

Nuclear extracts were prepared as previously described (Ikuyama, S., et al. (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al. (1993) J. Biol. Chem. 268, 24125-24137; Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Shimura, Y., et al. (1994) J. Biol. Chem. 269, 31908-31914, or by the procedure described in Example 8. For large scale preparations, FRTL-5 extracts were derived from cells grown to near confluency in 6H medium then maintained in 5H medium (-TSH), for 7 days. Cells were harvested by scraping, washed with Dulbecco's modified PBS without $Mg^{++}Ca^{++}$ (DPBS), pH 7.4, and, after centrifugation at 500xg, suspended in 5 pellet volumes of 0.3 M sucrose containing 2 % Tween-40, 10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A. After freezing, thawing, and gentle homogenization, nuclei

were isolated by centrifuging at 25,000xg on a 1.5 mM sucrose cushion containing the same buffer and lysed in 10 mM EGTA, 10 % glycerol, 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A. After centrifugation at 100,000xg for 1 h, the supernatant was dialyzed for use in gel mobility shift analyses.

Results

The 5'-decanucleotide in a tandem repeat (TR), -162 to -140 bp, of the TSH receptor (TSHR) promoter is in a CT-rich, S1 nuclease-sensitive region of the promoter (Ikuyama S., et al., (1992) Mol. Endocrinol., 6, 793-803; Ikuyama, S., et al., (1992) Mol. Endocrinol., 6, 1701-1715; Shimura, H., et al., (1993) J. Biol. Chem., 268, 24125-24137). A nonthyroid-specific factor binds the coding strand of the 5'-decanucleotide and decreases TSHR gene expression by suppressing the constitutive enhancer activity of the cAMP response element (CRE), -139 to -132 bp (Shimura, H., et al., (1993) J. Biol. Chem., 268, 24125-24137). A cDNA encoding the single-strand DNA-binding protein interacting with the 5'-decanucleotide (Figure 38A-B) and termed TSEP-1 has been cloned herein. Thus, clone 40 (Figure 38A), 1405 bp, encoded a protein with an open reading frame of 322 amino acids (Figure 38B). Sequence comparisons revealed that the protein encoded by the open reading frame was similar to a rat liver protein which was not characterized as a suppressor, but, rather as an enhancer: enhancer factor 1_A, $EF1_A$ (Ozer, J., et al., (1990) J. Biol. Chem., 265, 22143-22152; Faber, M., et al., (1990) J. Biol. Chem., 265, 22243-22254) or rat CBBF/CDS (Petty, K.J., et al., GenBank Accession Number M69138). EFI_A was identified by its ability to interact with the Rous sarcoma virus long terminal repeat enhancer and promoter at two inverted CCAAT box motifs. (Ozer, J., et al., (1990) J. Biol. Chem., 265, 22143-22152; Faber M., et al., (1990) J. Biol. Chem., 265, 22243-22254). Rat CBBP/CDS was cloned as a

protein whose binding was necessary for constitutive expression of the malic enzyme gene in the liver. TSEP-1 is approximately 95% identical to three human Y-box proteins. One is YB-1, a protein isolated from a lymphoblastoid cell line as a binding factor to the major histocompatibility complex class II inverted CCAAT motif, termed the Y-box, from which the family derives its name (Didier, D.K., et al., (1988) Proc. Natl. Acad. Sci. U.S.A., 85, 7322-7326). The second is DbpB, a protein nearly identical in sequence to YB-1 and cloned by its ability to bind inverted CCAAT motifs in the EGFR enhancer and the human c-erbB-2 enhancer (Sakura, H., et al. (1988) Gene, 73, 499-507). The third, either related to or identical to DbpB NSEP-1 (Kinniburgh, A.J. (1989) Nucleic Acid Res. 17, 7771-7778; Kolluri, R., et al., (1992) Nucleic Acid Res., 20, 111-116; Wolffe, A.P., (1992) New Biol., 4, 290-298; Kolluri, R., et al., (1991) Nucleic Acids Res. 19, 4771) which was cloned for its ability to bind CT-rich, nuclease sensitive, single strand binding elements of c-myc, EGFR, and Ki-ras.

These data indicated that the clone encoded a protein that is a member of the Y-box family of proteins (Wolffe, A.P., (1992) New Biol., 4, 290-298). One member of this family, human NSEP-1, has been previously cloned based on its ability to bind to single strand, CT-rich, S1 nuclease-sensitive promoter regions, similar to that associated with the 5'-decanucleotide of the TR. However, with the exception of human YB-1, which is a suppressor of MHC Class II genes (Ivashkiv, L.B., et al., (1991) J. Exp. Med., 174, 1583-1582; Ivashkiv, L.B., et al., (1994) Immunopharmacology, 27, 67-77; Vilen, B.J., et al., (1992) J. Biol. Chem., 267, 23728-23734; Brown, A.M., et al., (1993) J. Biol. Chem., 268, 26328-26333; Ting, J.P-Y, et al., (1994) J. Exp. Med., 179, 1605-1611; Wright, K.L., et al., (1994) EMBO J., 13, 4042-4053; MacDonald, G.H., et al., (1995) J. Biol. Chem., 270, 3527-353; Benoist, C., et

al., (1990) Annu. Rev. Immuno., 8, 681-715; Didier, D.K., et al., (1988) Proc. Natl. Acad. Sci. U.S.A., 85, 7322-7326), Y-box proteins are associated with enhancer rather than suppressor activity. By cotransfection with promoter-chloramphenicol acetyltransferase (CAT) chimeras containing the intact TR sequence, or inactivating mutations of each decanucleotide therein, we show that the protein regulates the function of the 5'-, but not the 3'-decanucleotide. (Figure 39A-39C).

This was determined when the cloned cDNA (Figures 38B-38B') was inserted into an expression vector, pRcCMV-TSEP-1, and was tested for its ability to decrease TSHR gene transcription by cotransfection with TSHR CAT chimeras containing the intact TR sequence, pTRCAT5'-177, or mutations of the 3', 5', or both decanucleotides (Figure 39A), pTRCAT5'-177mt1, pTRCAT5'-177mt2, and pTRCAT5'-177mt1+2, respectively (Shimura, H. et al., (1993) J. Biol. Chem. 268, 24125-24137). Mutation of each decanucleotide in the TR results in significantly higher CAT activity than exhibited by pTRCAT5'-177 (Figures 39B and 39C); mutation of both results in an additive increase in promoter activity (Figures, 39B and 39C), to levels comparable to pTRCAT5'-146, containing the CRE but not the TR (Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H., et al., (1993) J. Biol. Chem. 268, 24125-24137). Each decanucleotide acts, therefore, as a suppressor element. In 4 experiments, cotransfection of pRcCMV-TSEP-1 with pTRCAT5'-177, which contains both the 5'-and 3'-decanucleotides of the TR in their wild type form, decreased CAT activity (Figures 39B and 39C) to levels 50 ± 7% those of cotransfections with the pRc/CMV control vector. There was no effect of the pRcCMV-TSEP-1 expression vector or pRc/CMV on p8CAT, the vector used to construct pTRCAT5'-177. The Y-box protein that had been cloned was, therefore, a suppressor of the TSHR. More importantly, however, cotransfection of pRcCMV-TSEP-1

significantly (P<0.01) decreased pTRCAT5'-177mt1 activity, which has an intact 5'-decanucleotide sequence; the mean decrease in 4 experiments was to 45 ± 5% of control values. Cotransfection had no significant effect on the activity of pTRCAT5'-177mt2, which has the mutated 5'-decanucleotide sequence (Figures 38B and 39C). Further, cotransfection of pRcCMV-TSEP-1 with pTRCAT5'-177mt1+2, which has both the 5'-and 3'-decanucleotides of TR sequence mutated (Figure 39A), does not decrease CAT activity, the same as cotransfections with pRc/CMV (Figures 39B and 39C). These data indicated that the Y-box protein family member encoded by the cDNA in Figure 38 suppresses TSHR gene expression by interacting with the 5'-but not the 3'-decanucleotide of the TR site and has the functional suppressor characteristics predicted for TSEP-1, despite its role as an enhancer in the liver.

Using oligonucleotides with the same mutations, we show that the recombinant protein, termed TSEP-1 (TSHR suppressor element protein-1), forms a specific protein-DNA complex with the coding strand of the 5'-TR sequence, but not with noncoding or double strand DNA sequence.

Thus, confirming the functional data, the recombinant Y-box protein encoded by the full length clone in Figure 38 was shown to specifically bind the coding sequence of the 5'-decanucleotide of the TR. Recombinant His-tagged protein, produced in E. coli and affinity purified, is approximately 45 kDA by SDS-polyacrylamide gel electrophoresis (data not shown), consistent with the 42 kDa size of a Y-box protein (Spitkovsky, D.D., et al., (1992) Nucleic Acids Res. 20, 797-803) plus the His tag. In EMSA with ^{32}P-labeled single-and double-stranded probes, the recombinant protein was bound by the coding strand of the TR, ssTR2(+), the single-stranded oligonucleotide used as the probe for cloning. It did not, however, bind to oligonucleotides representing the noncoding [ssTR2(-)] or

double strand (dsTR2) of the TR. Further, the recombinant protein did not bind to single- or double-stranded TR1CRE probes, ssTR1CRE(+), ssTR1CRE(-), and dsTR1CRE, which contain the 3'-decanucleotide of the TR in a functional form (Ikuyoma, S. et al. (1992) Mol. Endocrinol. 6, 1701-1715; Shimura, H. et al. (1993) J. Biol. Chem. 268, 24125-24137) together with the CRE-like sequence of the TSHR promoter. These data support the conclusion that the member of the Y-box family encoded by the clone in Figure 38 acts as a suppressor of TSHR gene expression by interacting with the coding strand of the 5'-but not the 3'-decanucleotide of the TR.

Northern analyses indicate TSEP-1 is not thyroid-specific and is not TSH or insulin regulated. Thus, Northern analyses, using the radiolabeled insert from Clone 31 (Figure 38A) as a probe, revealed a 1.5 kb transcript in RNA preparations from rat FRTL-5 cells, as well as buffalo rat liver (BRL) cells and nonfunctional rat thyroid FRT cells. The mRNA size is, therefore, the same as that identified in the liver by rt CBBF/CDS (Petty, K.J., et al. GenBank Accession Number M69138 31). These data are consistent with our previous observation (Shimura, H., et al. (1993) J. Biol. Chem., 268:24125-24137) that the protein binding the 5'-decanucleotide was present in BRL cells and was not, therefore, thyroid-specific; they are consistent with the identification of TSEP-1 as a Y-box protein. Poly (A)+ RNA preparations from FRTL-5 rat thyroid cells maintained in the presence or absence of TSH had no significant difference in Y-box transcript levels. Removal of insulin/serum from the cell medium also did not change Y-box mRNA levels.

TSEP-1 is, therefore, a Y-box protein 95% identical both to human YB-1, which binds the Y-box of the major histocompatibility (MHC) class II gene and to human NSEP-1 (nuclease sensitive element protein 1), which binds single strand, CT-rich, nuclease-sensitive elements of

genes that, like the TSHR, have GC rich promoters: c-myc, the epidermal growth factor receptor, the insulin receptor, and Ki-ras (Ikuyama, S., et al., (1992) Mol. Endocrinol. 6, 793-803).

TSEP-1 binds two other sites in the minimal TSHR promoter in a single strand-specific fashion. One is associated with the insulin-response element of the minimal TSHR promoter and is not in an overtly CT-rich region. The other is located on the 3' end of the S-box of the TSHR, -120 to -113 bp, and is in a CT-rich area; TSEP-1 is a functional suppressor at each of these 2 sites (Figure 40A-C).

EMSA and oligonucleotide competition assays were used to determine other sites on the TSHR where TSEP-1 might interact. The formation of a protein/DNA complex between radiolabeled ssTR2(+) and nuclear extracts from FRTL-5 cells is prevented by the homologous unlabeled oligonucleotide, evidencing its specificity. Moving downstream, unlabeled TR1CRE, -153 to -114 bp, single or double strand, coding or noncoding, did not prevent complex formation when radiolabeled ssTR2(+) was used as probe (data not shown). However, an unlabeled single-stranded oligonucleotide which represents the coding strand sequence of the TSHR from -131 to -100 bp, termed ssS(+), was an effective inhibitor of radiolabeled ssTR2(+) complex formation. Unlabeled oligonucleotide, ssS(-), the noncoding strand counterpart of ssS(+), did not inhibit its formation nor did a double strand oligonucleotide encompassing this region of the TSHR.

Moving upstream, formation of the protein-DNA complex with radiolabeled ssTR2(+) was not inhibited by coding, noncoding, or double strand oligonucleotides representing the TSHR sequence between -194 and -169 bp. This region contains the thyroid transcription factor-1 (TTF-1) site and a single strand binding protein (SSBP) element on the noncoding strand, 5'- and contiguous with

the TTF-1 site; both of which are linked to maximal expression of the TSHR and TSH-cAMP-decreased TSHR gene expression (Shimura, H., et al., (1994) Mol. Endocrinol., 8, 1049-1069; Ohmori, M., et al., (1995) Endocrinology, 136, 269-282; Shimura, H., et al., (1995) Mol. Endocrinol., 9, 527-539). However, an oligonucleotide with the sequence of the noncoding but not the coding strand of the TSHR insulin response element, -220 to -188 bp (oligo TIF), was an effective inhibitor of ssTR2(+) complex formation. The double strand oligonucleotide was also not a competitor.

These data suggested there were two additional Y-box protein binding sites in the TSHR, both of which exhibited single strand specificity. One is below the CRE, between -131 and -100 bp. The other is in the region of the insulin response element, -220 to -188 bp. To confirm this, establish their functional role, and localize the sites, the following experiments were performed.

First, oligonucleotide competition using oligonucleotides containing these other sites as the radiolabeled probe was performed. Thus, the noncoding strand oligonucleotide representative of the region between -220 to -188 bp [oligo ssTIF(-)] was used as the radiolabeled probe and showed that a major protein-DNA complex was formed, whose mobility was identical to the ssTR2(+) complex formed with the same thyroid cell extracts. In addition, it was shown that its formation was prevented by the unlabeled coding strand of both TR2 [ssTR2(+)] and the S region [ssS(+)], but not their counterpart noncoding strands, ssTR2(-) or ssS(-). The same results were obtained in competition experiments using ssS(+) as the radiolabeled probe and unlabeled ssTR2(+) or ssTIF(-); unlabeled ssTR2(-) or ssTIF(+) were again not competitors (data not shown). Thus, converse competition experiments confirmed the existence of these

sites, their binding specificity, and their ability to form complexes of identical size, using the same cell extract.

Second, using radiolabeled oligonucleotides, we showed by direct binding that recombinant Y-box protein formed a complex with radiolabeled ssS(+) and ssTIF(-), but not radiolabeled ssS(-) or ssTIF(+). Also, neither radiolabeled double strand oligonucleotide, dsS or dsTIF, formed a complex with TSEP-1 protein. Thus, direct binding experiments with recombinant protein established that these were single strand, Y-box protein binding sites. In addition, they demonstrated that complex formation could be enhanced if the recombinant protein was preincubated with the catalytic subunit of protein kinase A plus ATP before adding radiolabeled probe. The PKA effect was duplicated with ssS(+), ssTR2(+) or ssTIF(-) as radiolabeled probes (data not shown). The effect was lost when the enzyme was boiled before use and was reversed by exposure to potato acid phosphatase but not albumin, the same as reported for TTF-1 (Shimura, H., et al., (1994) Mol. Endocrinol., 8, 1049-1069; Ohmori, M., et al., (1995) Endocrinology, 136, 269-282).

Third, cotransfection experiments with pRcCMV-TSEP-1 showed that both sites functioned as suppressor elements (Figure 40A-A'-40D-D'). Cotransfection with pTRCAT5'-220 into FRTL-5 cells can significantly ($p<0.05$) decrease CAT activity by comparison to cotransfection with control vectors alone (Figure 40A-A'). These data do not, however, prove that the region between -220 and -188 bp is a functional Y-box suppressor site, since this construct contains all three Y-box binding sites. To show this we cotransfected pRcCMV-TSEP-1 or the pRcCMV control with SV40-linked promoter constructs containing one or two copies of the insulin response element of the TSHR (Figure 40D). In each case cotransfection of the vector encoding the Y-box protein significantly suppressed promoter

activity (P<0.02) by comparison to cotransfection of pRcCMV. Cotransfection of pRcCMV-TSEP-1 into FRTL-5 (Figure 40B) or FRT (Figure 40C) cells reduced (P<0.05) the activity of pTRCAT5'-146 and pTRCAT5'-131, which have only the downstream Y-box protein binding site between -131 and -100 bp. In contrast, cotransfection had no effect on the activity of pTRCAT5'-90 or the p8CAT control which have no Y-box protein binding sites.

The inability of Y-box cotransfections to suppress pTRCAT5'-177mt1+2, but its ability to suppress pTRCAT5'-146 or pTRCAT5'-131, with the TR deleted, shows that the downstream S-box site is nonfunctional in the presence of a TR until after the Y-box protein binds to the 5' decanucleotide. Y-box protein binding to the 5' decanucleotide may, therefore, be a primary regulatory event and that the other Y-box protein binding sites, associated with the S-box and insulin response element, might become more available in a single strand, triplex DNA configuration, which the Y-box protein, NSEP-1, is suggested to promote after it binds to S1-nuclease sensitive, CT-rich regions on genes with GC-rich promoters (Kolluri, R., et al., (1992) Nucleic Acids Res. 20, 111-116).

Mutational analysis indicates that a conserved CCTC sequence in each TSEP-1 site is important for TSEP-1 binding and function. To better define the binding site for the Y-box protein in each locus, mutations were introduced into different portions of the ssS(+) or ssTIF(-) oligonucleotides. The ssS(+) mt1 oligonucleotide contains mutations in the 5' half of ssS(+), whereas the ssS(+)mt2 contains mutations in the 3'-half (Figures 32 and 34). The ssTIF(-)mt1 has mutations in the 5'-third of the wild type ssTIF(-), whereas the ssTIF(-)mt2 is mutated in the middle portion of the ssTIF(-) (Figures 32 and 34).

When radiolabeled and used as a probe, each mt1 oligonucleotide formed a complex with the recombinant Y-

box protein, which was identical in migration to that formed by the radiolabeled wild type probe. In contrast, a radiolabeled oligonucleotide with the mt2 mutations of both ssS(+) and ssTIF(-) lost the ability to form protein-DNA complexes with recombinant Y-box protein. The unlabeled mt2 mutant oligonucleotides also lost the ability to prevent complex formation between the radiolabeled wild type oligonucleotide and recombinant Y-box protein (data not shown).

Alignment of the ssTR2(+) sequence with the ssS(+) and ssTIF(-) regions where mutation resulted in a loss of Y-box protein binding identifies a conserved CCTC element in all locations. Mutation of the CCTC sequence in each site to GTAG resulted in a marked decrease in recombinant Y-box protein binding as evaluated by EMSA. These data indicate that a CCTC motif within each Y-box protein binding site, i.e. the 5' decanucleotide, the insulin response element region, and the S-box region of the TSHR, is critical for the single strand binding activity of the Y-box protein.

The S-box of the rat TSHR was so named because it has only a one base mismatch with the S-box of the murine Aα class II MHC gene and because, together with the X and Y-boxes, it is important in repression of constitutive class II gene expression. The functionally important consensus sequence of all class II S-boxes is CCTC/T. In sum, TSEP-1 is a Y-box protein suppressing constitutive TSHR gene expression by interacting with single-strand DNA binding sites in the rat TSHR promoter, one of which is the 5'-decanucleotide of the TR and is in a S1 nuclease-sensitive, CT-rich region of the TSHR minimal promoter. Another appears to be related to a site in the MHC class II promoter, which is involved in repression of that gene.

Expression of MHC class I is regulated during development and varies in different tissues (Ting, J. P-Y,

et al., (1993) Current Opinion in Immunology 5, 8-16); its precise regulation is crucial for the control of the immune response, since abnormally high levels are associated with autoimmune thyroid disease (ATD) and diabetes (Todd, I., et al., (1986) Annals N.Y. Acad. Sci., 475, 241-249). TTF-1 is a homeodomain protein which regulates thyroid development and the expression of genes associated with thyroid-specific function, i.e. the TSH receptor (TSHR) and thyroglobulin (TG) (Guazzi, S., et al., (1990) EMBO J. 9, 631-3639; Francis-Lang, H. et al., (1992) Mol. Cell. Biol. 12, 576-588; Zannini, M., et al., (1992) Mol. Cell. Biol. 12, 4230-4241; Shimura, H., et al. (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M., et al., (1995) Endocrinology, 136, 269-282; Kohn, L.D., et al., (1995) Vitamins and Hormones 50, 287-384). Its RNA levels are downregulated by TSH in thyroid cells. (Shimura, H., et al., (1994) Mol. Endocrinol. 8, 1049-1069; Ohmori, M. et al., (1995) Endocrinology 136, 269-282). A downstream 38 bp silencer, -127 to -89 bp in the MHC class I promoter, whose function depends on a cyclic AMP response element (CRE), -107 to -100 bp, and whose activity is regulated by TSH or methimazole (MMI) (Example 9) has been identified. Using gel shift assays (EMSA), it was shown that a protein/DNA complex formed by this silencer involves TTF-1, since it is present in rat thyroid, but not liver cells, and since its formation is inhibited by unlabeled oligonucleotides mimicking the TTF-1 binding sites on the TSHR and TG promoters (Figure 30). Using EMSA, recombinant TTF-1, and oligonucleotides mimicking -127 to -104 bp and -105 to -80 bp of the class I promoter, we identify two TTF-1 binding sites downstream and upstream of the CRE (Figure 41A); one is TTF-1-specific, the other can also interact with Pax-8. TTF-1 and CRE binding protein, CREB, footprint the region between -120 to -89 bp and -113 to -95 bp, respectively;

the overlapping footprints shows that TTF-1 and CREB binding is mutually competitive.

Overexpression of TTF-1 in rat thyroid cells increases the activity of a class I-reporter gene chimera containing the TTF-1 sites and the CRE, p(-209)CAT or p(-127)CAT (Figure 41C), but not the activity of a chimera without them, p(-68)CAT, nor a chimera with a nonpalindromic CRE mutation, p(-209NPCRE). In contrast, overexpression of TSEP-1 or Y-box cDNA decreases Class I promoter activity (Figure 41C). When TSH decreases class I levels, it coordinately decreases TTF-1 complex formation with the silencer and increases complex formation with 2 Y-box protein (TSEP-1) suppressor sites, one near each TTF-1 element (Figure 41A). MMI will also decrease TTF-1 mRNA levels (Table VIII); MMI will also reverse the ability of interferon to decrease TSEP-1 RNA levels (Table IX). TSEP-1 is a suppressor of class I activity when cotransfected with class I promoter-reporter gene chimeras (Figure 41C). Using gel shift assays and oligonucleotide competitors from the TSHR gene, we identify 2 TSEP-1 sites on the coding strand of the class I promoter, surrounding the CRE. One is 3' to the CRE and within the 48 bp silencer; the other is upstream, between the insulin response element and CRE. The TSEP-1 sites are in each case associated with thyroid transcription factor-1 (TTF-1) elements. Mutation data indicate TTF-1/TSEP-1 binding to their respective sites is mutually exclusive. Interferon (IFN) increases class I expression in thyroid cells; MMI reverses this (M. Saji et al., (1992b)). IFN decreases TSEP-1 RNA levels (Table IX) and complex formation with this region of the class I promoter (data not shown); MMI reverses this (Table IX). In sum, TSEP-1 is a negative regulator of MHC class I and TSHR gene expression.

TABLE VIII

Effect of MMI on TTF-1 RNA levels

TREATMENT	TTF-1 RNA LEVEL (% of Control with no treatment)
NONE	100%
MMI 5 mM	32%

Figure Legend for Table VIII. FRTL-5 thyroid cells were maintained in medium without TSH for 6 days after being grown to 80% confluency. At the start of the experiment, cells were exposed to fresh medium with or without 5 mM MMI. After 24 hours, cells were harvested, RNA isolated, and Northern analyses performed using the cDNA for TTF-1 as described (Shimura, H., et al., (1994) Mol. Endocrinol. 8:1049-1069; Ohmori, M., et al., (1995) Endocrinology 136:269-282). Quantitation was by densitometry; the TTF-1 level with no cell treatment was set at 100%.

TABLE IX

Effect of MMI on interferon-induced decreases in TSEP-1 (Y-box) RNA levels

TREATMENT	TSEP-1 RNA LEVEL (% of Control with no treatment)
NONE	100%
MMI 5 mM	100% ± 7%
γ INTERFERON 100 Units	29 ± 9%
γ INTERFERON 100 Units Plus MMI 5 mM	106 ± 10%

Figure Legend for Table IX. FRTL-5 thyroid cells were maintained in medium without TSH for 6 days after being grown to 80% confluency. At the start of the experiment, cells were exposed to fresh medium with or without interferon and/or 5mM MMI. After 24 hours, cells were harvested, RNA isolated, and Northern analyses performed using the clone 40 insert (Figure 38). Analyses were performed as in Table VIII (Shimura, H., et al., (1994) Mol. Endocrinol. 8:1049-1069; Ohmori, M., et al., (1995) Endocrinology 136:269-282). Quantitation was by densitometry; the TESP-1 level in cells with no cell treatment was set at 100%.

In sum, TTF-1 is a positive regulator of MHC class I gene expression in thyroid cells by its action on a downstream 38 bp silencer. TSH and MMI decrease class I expression by decreasing TTF-1 RNA and protein levels, thereby decreasing TTF-1 positive regulation. TTF-1 and class I expression are, therefore, normally coregulated by TSH and MMI.

TTF-1 interactions with this silencer are normally coordinated with TSEP-1. TSEP-1 normally suppresses MHC class I, and suppression is eliminated by interferon. TSEP-1 binding, activity and suppression can be returned to normal by MMI. Thus, the downstream silencer is a region of tissue-specific control, normally regulated by TSEP-1/TTF-1, subject to abnormal regulation in autoimmune thyroid disease, and a site of action for MMI.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTATCT TTCCTAATTA CCATTCTTCA ATCCATACTT 40

TAATAGTATT GTCTCTGAGG ACGTAGGAAG TACATATGAA 80

ACACTCCTGC TACCTTCCAA AGTACTGTGT CCCAAGGAAA 120

ATCATTCTGT GAGCTGCACT AGCCTCTTTT TCATGGAATA 160

CAACCTTTAC TGGAAAGAAT GAATGACACT GGAAGATCTA 200

TATAACTTAG TGAAACAATG TATTCGGTCT TAAAACTCTT 240

ACATTAGTAT AAGCAACAGT CAATGTGCAA GCCAGGCTTT 280

TAATTTAACA GAATAGGAAA CACGGAGTAT ACTGATTCAG 320

GTCCACATTC AAAATAACCT TTGAGAAATT ACCATTATGA 360

TAGCATCCAA AATTATCTGA AAAGGTTATT AAAAATACAT 400

GTCCTACATG TGTGCGGGGC TTTTACATTT CATAGATGTC 440

AGCCACCAAA AGGACTCAGC ACAGAAGCAG ACATAAACCT 480

CCAGTGGTTT TCCCATGAGC CAGACAGCAG AGAGACTTGC 520

CATAGAGTAA AATGTAAAAA GCTCCACTCT TCACACTACA 560

GTGTTTCTTA TGCGAAATAA TTGTTTTCAT ATGAAATGCA 600

TGGATTATTT ATATCTTCTA AAAATTTGAT GAAATTTTAA 640

ACTATTATTT CTAGTATAGA AAATATCCAC TGACGTATCA 680

ACACAAACAT ATCTTAGAGG TCTTCACTAA TTTGTAAAAC 720

TGTAGGAATA TTCTCACTAA AAGGTTTGGA AATCGCTGGG 760

TACACAGCCC CTGGGCCACT GGAGGCACTG GAGACACTGT 800

GACAAAGAGC TTTCTGAAGA GCAGCAGGGC AGAGTCCCAG 840

CTCCGCAGCC AGGCGTGGCT CTCAGGGTCT CAGGCTCCAG 880

GGCGGAGTCT GGGCGGGGAG GCGCGGTGGT GGGGAGTCCC 920

CGTGTCCCCA GTTTCACTTC TCCGTCTCGC AACCTGTGTG 960

GGACCGTCCT GCCCGGACAC TCGTGACGCG ACCCCACTTC 1000

TCTCTCCTAT TGCGTGTCCG GTTTCTGGAG AAGCCAATCG 1040

GCGCCACTGC GGTTCCCGGT TCTAAACTCT CCACCCACCC 1080

GGCTCTGCTC AGCTTCTCCC CAGACTCCGA GGCTGAGGAT 1120

C ATG GGG CCT GGA GCC CTC TTC CTG CTG CTG TCG 1154
Met Gly Pro Gly Ala Leu Phe Leu Leu Ser
1 5 10

GGA ACC TTG GCC CTG ACC GGC ACC AAG GCG GGT 1187
Gly Thr Leu Ala Leu Thr Gly Thr Lys Ala Gly
15 20

GAGTGCGGGA TCGGGAACAA GGCCGCTGCG GGGAGGAGCT 1227

GAGGCACCGC CTGGGAGTCG GGTGGGGGCA GGACCCACGG 1267

GGAAGGTGCG ACTCTGCTGT CCCGGCCCAG ACCCGCCACC 1307

TCACCCCGTC CTGTCCTGTC CCTCCCTTGC TTCCTGCTCC 1347

TCTGCTTTTC CCCCCTAAAC CCGGGGCCCG TCTCCGACCT 1387

CCACCCCTTT CCCGCCTCCC GAGCCCCGAG CT 1419

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTCCACATT CAAAATAACC TTTGAGAAAT TACCATAATG 40

ATAGCATCCA AAATTATCTG AAAAGGTTAT TAAAAATACA 80

TGTCCTACAT GTGTGCGGG 99

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGCGAATGAT AGCATCCAAA ATTATCTGAA AAGGTTAGCG 40

C 41

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGCCAAAATT ATCTGAAAAG GTTATTAAAA ATACATGTCG 40

G 41

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCCAAAATT ATCTGAAAAG GTTATTAAAA GCGC 34

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34
(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGCCTGGTAA TTTCTCAAAG GTTATTAAAA GCGC 34

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGCCAAAATT ATCTGAAACT CGCGTTTTGA GCGC 34

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGCCAAAATT ATTCTCATAG GGTATTAAAA GCGC 34

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTCAAAAGGT TATTAAAAAT GTGGC 25

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE

(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCGTTAAAA ATACATGTCC TACATGTGTG C 31

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGCGATGGTA ATTTCTCAAA GGTTATTTTG AATGTGGTCC 40

GG 42

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCGCAAAGGT TATTTTGAAT GTGGACCGG 29

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCGCAAAGGT TCGGTTGAAT GTGGACCGG 29

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29

(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCGCAAAGTG GATTTTGAAT GTGGACCGG 29

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCGCTCCTGT TCGGTTGAAT GTGGACCGG 29

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGCCAAAGGT TATTTTGAAA CTGGC 25

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGCCTGGTAA ATCTGAAAAG GTCGTTTTGA GCG 33

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30
(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTTACACACG ATGTGCATAT TAGGACATCT 30

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTAGGACATG TATTTTTAAT AACCTTTTCA GATAATTTT 39

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGTCCACATT CAAAATAACC TTTGAGAAAT TACCATCGCG 40

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGTCCACATT CAAAATAACC TTTGCGC 27

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE

(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGTCCACATT CAAAATCCAC TTTGCGC 27

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GCCACATTTT TAATAACCTT TTGAG 25

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTCCACATTC AAAATAACAG GAGCGC 26

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCGGACATGT ATTTTTAATA ACCTTTTCAG ATAATTTTGG 40

CC 42

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34
(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GCGCTTTTAA TAACCTTTTC AGATAATTTT GGCC 34

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GCGCTTTTAA TAACCTTTGA GAAATTACCA GGCC 34

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCGCTTTTAA TACCCTATGA GAATAATTTT GGCC 34

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CGCGTTCAAA ATAACCTTTG GCC 23

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE

(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGTCCACATT CAACCGAACC TTTGCGC 27

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGTCCACATT CAACCGAACA GGAGCGC 27

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GCCAGTTTCA AAATAACCTT TGGCC 25

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GTAGGACATG TATTTTTAAC GCG 23

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GCGCTCAAAA CGCGAGTTTC AGATAATTTT GGCC 34

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 106
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CATATGAAAT GCATGGATTA TTTATATCTT CTAAAAATTT 40

GATGAAATTT TAAACTATTA TTTCTAGTAT AGAAAATATC 80

CACTGACGTA TCAACACAAA CATATC 106

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TGAAACAATG TATTCGGTCT AAAACTCTTA CATTAGTATA 40

AGCAACAGTC AATGTGCAAG CCAGGCTTTT AATTTAACAG 80

AATAGGAAAC ACGGAGTATA CTGATTCAG 109

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 135
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GTCCACATTC AAAATAACCT TTGAGAAATT ACCATAATGA 40

TAGCATCCAA AATTATCTGA AAAGGTTATT AAAAATACAT 80

GTCCTACATG TGTGCGGGGC TTTTACATTT CATAGATGTC 120

AGCCACCAAA AGGAC 135

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TGACTAGCAG AGAAAACAAA GTGA 24

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1422
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AAAGCCGGGC CATGGTACAA CAGACCAACA ACGCCGAGAA 40

CACGGAGGCT TTGCTGGCTG GGGAGAGCTC AGACTCGGGC 80

GGCGGCCTGG AGCTGGGCAT CGCGTCCTCC CCGACGCCCG 120

GCTCCACGGC GTCCACGGGT GGCAAGGCGG ACGACCCTAG 160

CTGGTGCAAG ACGCCCAGTG GCCACATCAA GCGGCCCATG 200

AACGCCTTTA TGGTGTGGTC GCAGATCGAG CGGCGCAAGA 240

TCATGGAGCA GTCGCCCGAC ATGCACAACG CCGAGATCTC 280

CAAGCGGCTG GGCAAACGCT GGAAGCTGCT CAAGGACAGC 320

GACAAGATCC CGTTCATCCA GGAGGCGGAG CGGCTGCGCC 360

TCAAGCACAT GGCTGACTAC CCTGACTACA AGTACCGGCC 400

GCGAAAGAAG GTGAAGTCGG GCAACACGGG CGCGGGATCG 440

GCGGCCACAG CCAAACCTGG GGAGAAGGGC GACAAGGTCG 480

GCGGCGGCAG CGGCCACGCG GGAAGCGGCC ACGCGGGGGG 520

TGGCGCGGGC GGCAGCTCCA AGCCCGCGCC CAAGAAGAGC 560

TGCGGCCCCA AGGTGGCGGG CAGCTCGGTC GGCAAGCCCC 600

ACGCCAAGTT CGTCCCGGCG GGCGGCGGTA AGGCGGCTGC 640

ATCGTTCTCT CCGGAGCAGG CCGCCCTGCT GCCCCTGGGG 680

GAGCCCGCGG CCGTCTACAA GGTGCGGACT CCCAGCGCGG 720

CCACCCCGGC CGCCTCCTCC TCGCCGTCCA GCGCGCTGGC 760

CACCCCCGCC AAACACCCTG CCGACAAGAA GGTGAAGCGC 800

GTTTACCTGT TCGGAAGCCT GGGCGCTTCG GCATCCCCGG 840

TCGGGGGCCT GGGAGCGAGC GCTGACCCCA GCGATCCACT 880

GGGGTTATAC GAAGATGGGG GCCCGGGATG CTCGCCCGAT 920

GGCCGGAGTC TGAGCGGCCG TAGCAGCGCA GCATCATCGC 960

CCGCCGCCAG CCGATCGCCC GCTGACCACC GCGGCTACGC 1000

CAGCCTACGT GCAGCCTCGC CCGCCCCGTC CAGCGCGCCC 1040

TCGCACGCGT CCTCGTCGCT CTCCTCATCC TCCTCCTCTT 1080

CCTCGGGCTC TTCTTCGTCC GATGATGAGT TCGAAGATGA 1120

CCTGCTCGAC CTGAACCCCA TCTCAAACTT TGAGAGCATG 1160

TCCCTGGGCA GTTTCAGCTC CTCATCCGCT CTTGATCGGG 1200

ACCTGGATTT TAACTTCGAA CCCGGCTCAG GCTCCCACTT 1240

CGAGTTCCCG GACTATTGCA CGCCCGAGGT GAGCGAGATG 1280

ATCTCGGGAG ATTGGCTGGA GTCCAGCATC TCTAACCTGG 1320

TCTTCACCTA CTGAAGGGAG CGCGGGCCGG GGAGAAGGAG 1360

GGCCAAGAGG CAGGAGAGGA GAGAGGAAGA CAAAAAACAA 1400

AACAAAACAA AAATCGGAAT TC 1422

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 440

(B) TYPE: AMINO ACID
(C) STRANDEDNESS: UNKNOWN
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```
Met Val Gln Gln Thr Asn Asn Ala Glu Asn Thr Glu
 1               5                  10
Ala Leu Leu Ala Gly Glu Ser Ser Asp Ser Gly Ala
            15                  20
Gly Leu Glu Leu Gly Ile Ala Ser Ser Pro Thr Pro
 25                  30                  35
Gly Ser Thr Ala Ser Thr Gly Gly Lys Ala Asp Asp
                40                  45
Pro Ser Trp Cys Lys Thr Pro Ser Gly His Ile Lys
     50                  55                  60
Arg Pro Met Asn Ala Phe Met Val Trp Ser Gln Ile
                65                  70
Glu Arg Arg Lys Ile Met Glu Gln Ser Pro Asp Met
            75                  80
His Asn Ala Glu Ile Ser Lys Arg Leu Gly Lys Arg
 85                  90                  95
Trp Lys Leu Leu Lys Asp Ser Asp Lys Ile Pro Phe
            100                 105
Ile Gln Glu Ala Glu Arg Leu Arg Leu Lys His Met
    110                 115                 120
Ala Asp Tyr Pro Asp Tyr Lys Tyr Arg Pro Arg Lys
                125                 130
Lys Val Lys Ser Gly Asn Thr Gly Ala Gly Ser Ala
            135                 140
Ala Thr Ala Lys Pro Gly Glu Lys Gly Asp Lys Val
145                 150                 155
Gly Gly Gly Ser Gly His Ala Gly Ser Gly His Ala
            160                 165
Gly Gly Gly Ala Gly Gly Ser Ser Lys Pro Ala Pro
    170                 175                 180
Lys Lys Ser Cys Gly Pro Lys Val Ala Gly Ser Ser
                185                 190
Val Gly Lys Pro His Ala Lys Phe Val Pro Ala Gly
            195                 200
Gly Gly Lys Ala Ala Ala Ser Phe Ser Pro Glu Gln
205                 210                 215
Ala Ala Leu Leu Pro Leu Gly Glu Pro Ala Ala Val
            220                 225
Tyr Lys Val Arg Thr Pro Ser Ala Ala Thr Pro Ala
    230                 235                 240
Ala Ser Ser Ser Pro Ser Ser Ala Leu Ala Thr Pro
                245                 250
Ala Lys His Pro Ala Asp Lys Lys Val Lys Arg Val
            255                 260
Tyr Leu Phe Gly Ser Leu Gly Ala Ser Ala Ser Pro
265                 270                 275
```

```
Val Gly Gly Leu Gly Ala Ser Ala Asp Pro Ser Asp
            280                 285
Pro Leu Gly Leu Tyr Glu Asp Gly Gly Pro Gly Cys
    290                 295                 300
Ser Pro Asp Gly Arg Ser Leu Ser Gly Arg Ser Ser
                305                 310
Ala Ala Ser Ser Pro Ala Ala Ser Arg Ser Pro Ala
        315                 320
Asp His Arg Gly Tyr Ala Ser Leu Arg Ala Ala Ser
325                 330                 335
Pro Ala Pro Ser Ser Ala Pro Ser His Ala Ser Ser
          340                 345
Ser Leu Ser Ser Ser Ser Ser Ser Ser Ser Gly Ser
    350                 355                 360
Ser Ser Ser Asp Asp Glu Phe Glu Asp Asp Leu Leu
                365                 370
Asp Leu Asn Pro Ser Ser Asn Phe Glu Ser Met Ser
        375                 380
Leu Gly Ser Phe Ser Ser Ser Ser Ala Leu Asp Arg
385                 390                 395
Asp Leu Asp Phe Asn Phe Glu Pro Gly Ser Gly Ser
            400                 405
His Phe Glu Phe Pro Asp Tyr Cys Thr Pro Glu Val
    410                 415                 420
Ser Glu Met Ile Ser Gly Asp Trp Leu Glu Ser Ser
                425                 430
Ile Ser Asn Leu Val Phe Thr Tyr
        435                 440
```

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1512
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

```
GAATTCCGGT CTCACTGGTC TACCTTGCTC TCCTGCACCC          40

TGGTTGTCAG CACCCACCAT CACACCCGGG AGGAGCCGCA          80

GCCGTCGCCG CCGGCCCCAG TCACCATCAC CGCAACCATG         120

AGCAGCGAGG CCGAGACCCA GCAGCCGCCC GCCGCCCCCG         160

CCGCCGCCCT CAGCGCCGCC GACACCAAGC CCGGCTCCAC         200

GGGCAGCGGC GCGGGTAGTG GCGGCCCGGG CGGCCTCACA         240

TCGGCGGCGC CCGCCGGCGG GGACAAGAAG GTCATCGCAA         280
```

CGAAGGTTTT GGGAACAGTA AAATGGTTCA ATGTAAGGAA 320

CGGATACGGT TTCATCAACA GGAATGACAC CAAGGAAGAC 360

GTATTTGTAC ACCAGACGGC CATAAAGAAG AATAACCCCA 400

GGAAGTACCT TCGCAGTGTA GGAGATGGAG AGACTGTGGA 440

GTTTGATGTT GTTGAAGGAG AAAAGGGTGC GGAGGCAGCT 480

AATGTTACAG GCCCTGGTGG AGTTCCAGTT CAAGGCAGTA 520

AATACGCAGC AGACCGTAAC CATTATAGGC GCTATCCACG 560

TCGTAGGGGT CCTCCACGCA ATTACCAGCA AAATTACCAG 600

AATAGTGAGA GTGGGGAAAA GAATGAAGGA TCGGAAAGCG 640

CTCCTGAAGG CCAGGCCCAA CAACGCCGGC CCTATCGCAG 680

CCGAAGGTTC CCACCTTACT ACATGCGGAG GCCCTATGCG 720

CGTCGACCAC AGTATTCCAA CCCCCCTGTG CAAGGAGAAG 760

TGATGGAGGG TGCTGACAAC CAGGGTGCAG GAGAGCAAGG 800

TAGACCAGTG AGACAGAATA TGTATCGGGG TTACAGACCA 840

CGATTCCGCA GGGGCCCTCC TCGCCCAAGA CAGCCTAGAG 880

AGGATGGCAA TGAAGAGGAC AAAGAAAATC AAGGAGATGA 920

GACCCAAGGT CAGCAGCCAC CTCAACGTCG GTATCGCCGC 960

AACTTCAATT ACCGACGCAG ACGCCCAGAG AACCCTAAAC 1000

CACAAGATGG CAAAGAGACA AAAGCAGCCG ATCCACCAGC 1040

TGAGAATTCG TCCGCTCCCG AGGCTGAGCA GGGCGGGGCT 1080

GAGTAAATGC CGGCTTACCA TCTCTACCAT CATCCGGTTT 1120

GGTCATCCAA CAAGAAGAAA TGAATATGAA ATTCCAGCAA 1160

TAAGAAATGA ACAAAGATTG GAGCTGAAGA CCTTAAGTGC 1200

TTGCTTTTTG CCCGTTGACC AGATCCACTA GAACTGTCTG 1240

CATTATCTAT GCAGCATGGG GTTTTTATTA TTTTTACCTA 1280

AAGATGTCTC TTTTTGGTAA TGACAAACGT GTTTTTTAAG 1320

AAAAAAAAAA AGGCCTGGTT TTTCTCAATA CACCTTTAAC 1360

GGTTTTTAAA TTGTTTCATA TCTGGTCAAG TTGAGATTTT 1400

TAAGAACTTC ATTTTTAATT TGTAATAAAG TTTACAACTT 1440

GATTTTTTCA AAAAAGTCAA CAAACTGCAA GCACCTGTTA 1480

ATAAAGGTCT TAAATAATAA AAAACGGAAT TC 1512

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 322
(B) TYPE: AMINO ACID
(C) STRANDEDNESS: UNKNOWN
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Met Ser Ser Glu Ala Glu Thr Gln Gln Pro Pro Ala
1 5 10
Ala Pro Ala Ala Ala Leu Ser Ala Ala Asp Thr Lys
15 20
Pro Gly Ser Thr Gly Ser Gly Ala Gly Ser Gly Gly
25 30 35
Pro Gly Gly Leu Thr Ser Ala Ala Pro Ala Gly Gly
40 45
Asp Lys Lys Val Ile Ala Thr Lys Val Leu Gly Thr
50 55 60
Val Lys Trp Phe Asn Val Arg Asn Gly Tyr Gly Phe
65 70
Ile Asn Arg Asn Asp Thr Lys Glu Asp Val Phe Val
75 80
His Gln Thr Ala Ile Lys Lys Asn Asn Pro Arg Lys
85 90 95
Tyr Leu Arg Ser Val Gly Asp Gly Glu Thr Val Glu
100 105
Phe Asp Val Val Glu Gly Glu Lys Gly Ala Glu Ala
110 115 120
Ala Asn Val Thr Gly Pro Gly Gly Val Pro Val Gln
125 130
Gly Ser Lys Tyr Ala Ala Asp Arg Asn His Tyr Arg
135 140
Arg Tyr Pro Arg Arg Arg Gly Pro Pro Arg Asn Tyr
145 150 155
Gln Gln Asn Tyr Gln Asn Ser Glu Ser Gly Glu Lys
160 165
Asn Glu Gly Ser Glu Ser Ala Pro Glu Gly Gln Ala
170 175 180
Gln Gln Arg Arg Pro Tyr Arg Arg Arg Arg Phe Pro
185 190

```
Pro Tyr Tyr Met Arg Arg Pro Tyr Ala Arg Arg Pro
        195                 200
Gln Tyr Ser Asn Pro Pro Val Gln Gly Glu Val Met
205                 210                 215
Glu Gly Ala Asp Asn Gln Gly Ala Gly Glu Gln Gly
            220                 225
Arg Pro Val Arg Gln Asn Met Tyr Arg Gly Tyr Arg
    230                 235                 240
Pro Arg Phe Arg Arg Gly Pro Pro Arg Gln Arg Gln
                245                 250
Pro Arg Glu Asp Gly Asn Glu Glu Asp Lys Glu Asn
        255                 260
Gln Gly Asp Glu Thr Gln Gly Gln Gln Pro Pro Gln
265                 270                 275
Arg Arg Tyr Arg Arg Asn Phe Asn Tyr Arg Arg Arg
            280                 285
Arg Pro Glu Asn Pro Lys Pro Gln Asp Gly Lys Glu
    290                 295                 300
Thr Lys Ala Ala Asp Pro Pro Ala Glu Asn Ser Ser
                305                 310
Ala Pro Glu Ala Glu Gln Gly Gly Ala Glu
        315                 320
```

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CTTGTTTGGA GAGTTGCCTA GGCAAGCGG 29

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CTTGTTTGGG GCATTGCCTA GGGAAGCGG 29

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CTTATGTAGA GAGTTGCCTA GGCAAGCGG 29

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

AAGCGGAGCA CTTGAGAGCC TCTCC 25

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

AAGCGAAACG CCAGTGCGAC TCTCC 25

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CACTGCCCAG TCAAGTGTTC TTG 23

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22

(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CACTGCCCAG ATTCTGTTCT TG 22

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GTTCGCCTCG TGAACTCTCG GAGAGG 26

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

AGCCTCTCCT TCCCCCTTCC CCCTCTCCAG CGTGCTCTCC 40

AGCGATG 47

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

AGCCTCTCCT TCCCCCTCTC CAGCGTGCTC TCCAGCGATG 40

AGGTCA 46

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

AGCCTCTCCT TCCCCCTCTC CAGCGTGTTC ACCTGTGATG 40

AGGTCA 46

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

AGCCTCTCCT TCCCCCACTG CATCTTGCTC TCCAGCGATG 40

AGGTCA 46

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CAGCCCCTTG GAGCCCTCCT CCTTCCTCCC TT 32

(2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CAGCCGCTAG TAGGCCTCCT CCTTCCTCCC TT 32

(2) INFORMATION FOR SEQ ID NO: 57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

CAGCCCCTTG GAGCCCTCCA CGTTGCTCCC TT 32

(2) INFORMATION FOR SEQ ID NO: 58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

GAACAAACCT ACCTCTCAAC GGATCCGTTC GCC 33

(2) INFORMATION FOR SEQ ID NO: 59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

GAATACATCT ACCTCTCAAC GGATCCGTTC GCC 33

(2) INFORMATION FOR SEQ ID NO: 60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GAACAAACCC AGTTCCAACG GATCCCTTCG CC 32

(2) INFORMATION FOR SEQ ID NO: 61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

GTTCGCCTCG TGAACTCTCG GAGAGG 26

(2) INFORMATION FOR SEQ ID NO: 62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

GTTCACCTCC TGAACTCTCG GAGAGG 26

(2) INFORMATION FOR SEQ ID NO: 63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

AGCCTCTCCT TCCCCCTCTC CAGCGTGTTC ACCTGTGATG 40

AGGTCA 46

(2) INFORMATION FOR SEQ ID NO: 64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

AGCCTCTCCT TCCCCCACTG CATCTTGCTC TCCAGCGATG 40

AGGTCA 46

(2) INFORMATION FOR SEQ ID NO: 65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

AGCCTCTCCT TCCCCCACTG CATCTTGTTC ACCTGTGATG 40

AGGTCA 46

(2) INFORMATION FOR SEQ ID NO: 66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

AGCCTCTCCT TCCCCCTCTC CAGCGTGCTC TCCAGCGATG 40

AGGTCA 46

We Claim:

1. A method for assessing the ability of a drug to suppress expression of MHC Class I molecules by measuring altered binding of a mammalian cell protein extract to a MHC Class I regulatory nucleic acid sequence or functional equivalents thereof, said method comprising the steps of:

(a) treating the mammalian cells with said drug;

(b) obtaining protein extract from said cells in step (a) and combining said extract with a MHC Class I regulatory nucleic acid sequence to allow formation of at least one complex between said protein and said sequence; and

(c) detecting said complex; wherein altered formation of said complex indicates said drug's potential in treating autoimmune diseases or transplantation rejection.

2. The method of claim 1 wherein said autoimmune disease is a non-thyroid autoimmune disease.

3. The method of claim 1, wherein said protein forming said complex comprises at least one protein selected form the group consisting of NF-κ B and its subunits, c-fos family members or related proteins, a Sox-4 protein, a Y-box protein, a Pax 8 protein, a single stranded binding protein (SSBP), and a cyclic AMP regulatory binding protein (CREB) or c-jun family member, or functional equivalents thereof.

4. The method of claim 1 wherein said regulatory nucleic acid sequence is selected from the group consisting of enhancer sequence, silencer sequence or functional equivalents thereof.

5. The method of claim 1 wherein said regulatory nucleic acid sequence is selected from the PD1 regulatory domain between about -127 to about -90 base pairs, about -127 to about -80 base pairs, about -724 to about -697 base pairs or functional equivalents thereof.

6. The method of claim 5, wherein said regulatory nucleic acid sequence is selected from the group consisting of the PD1 promoter fragments designated 140 (bases 321 to 455 of SEQ ID NO:1), 114 (bases 221 to 320 of SEQ ID NO:1), 151 (bases 54 to 220 of SEQ ID NO:1) and 238 (bases 456 to 692 of SEQ ID NO:1) or functional equivalents thereof.

7. The method of claim 1 wherein said nucleic acid sequences are double or single stranded oligo nucleotides.

8. The method of claim 1, wherein said regulatory nucleic acid sequence is selected from the group consisting of the oligonucleotides designated S1 (SEQ ID NO:3), S2 (SEQ ID NO:4), S3 (SEQ ID NO:10), S5 (SEQ ID NO:5), S6 (SEQ ID NO:6), S7 (SEQ ID NO:7), S8 (SEQ ID NO:8) and the functional equivalents thereof.

9. The method of claim 1, further comprising the step of adding oligo nucleotide K to step (b).

10. A method for assessing the ability of a drug to suppress expression of MHC Class I molecules by measuring decreased activity of a reporter gene operably linked to a MHC Class I regulatory nucleic acid sequence, said method comprising:

(a) introducing said reporter gene operably linked to said MHC Class I regulatory sequence into a population of mammalian cells;

(b) treating said cells with said suppressing drug; and

(c) measuring activity of said reporter gene attached to said MHC Class I regulatory sequence; whereby said decreased activity is indicative of said drugs ability to suppress MHC Class I molecules.

11. The method of claim 10 wherein said regulatory nucleic acid sequence is an enhancer or silencer sequence.

12. The method of claim 10 wherein said regulatory nucleic acid sequence sequences is an upstream, or downstream silencer of the PD1 gene, or an upstream or downstream enhancer of the PD1 gene, or functional equivalents thereof.

13. The method of claim 10, wherein said reporter gene is chloramphenicol acetyltransferase (CAT) gene, the β-galactosidase gene, the luciferase gene or human growth hormone (hGH) or functional equivalents thereof.

14. A method of assessing the therapeutic potential of a candidate drug by detecting messenger RNA for genes capable of modulating MHC Class I expression in a sample comprising the steps of:

(i) treating cells with said candidate drug;

(ii) contacting all or part of a nucleic acid sequence encoding for a protein capable of modulating MHC Class I expression with said sample under conditions allowing a complex to form between said nucleic acid sequence and said messenger RNA from said treated cells;

(iii) detecting said complexes; and

(iv) determining the level of said messenger RNA; whereby an alteration in said mRNA is indicative of said drug's therapeutic potential for treating autoimmune disease or transplantation rejection.

15. The method of claim 14 wherein said altered level is a decreased or increased level of mRNA.

16. The method of claim 14 wherein said mRNA encodes for a Sox-4 protein, TTF-1 protein, SSBP protein, a Y-Box protein or functional equivalents thereof.

17. A method of assessing the therapeutic potential of a candidate drug for treating autoimmune disease or transplantation rejection by detecting a protein capable of modulating MHC Class I expression comprising the steps of:

(i) treating said cells with a candidate drug;

(ii) contacting a reagent which specifically reacts and forms a complex with said protein from said treated cells which is capable of modulating MHC Class I expression; and

(iii) detecting the formation of said complex with protein from said treated cells and said reagent, whereby alterations in the level of MHC Class I expression is indicative of said drugs therapeutic value on treating autoimmune disease or transplantation rejection.

18. A method for assessing the therapeutic potential of a candidate drug for treating autoimmune disease or transplantation rejection by assessing the

ability of said candidate drug to suppress MHC Class I molecules, said method comprising:

(a) treating cells with said candidate drug; and

(b) assessing the oxidation/reduction state of proteins from said treated cell which are capable of modulating MHC Class I expression; wherein an alteration is said protein oxidation/reduction state is indicative of said drug's therapeutic value in treating autoimmune disease or transplantation rejection.

19. The method of claim 18 wherein said protein capable of modulating MHC Class I expression is TTF-1, Sox-4, Y-box, SSBP, NF-κB, e-fos, c-jun, or their functional equivalents.

20. A method for assessing the therapeutic potential of a candidate drug for treating autoimmune disease or transplantation rejection by assessing the ability of said candidate drug to suppress MHC Class I molecules, said method comprising:

(a) treating cells with said candidate drug; and

(b) assessing the activity of an enzyme responsible for oxidation/reduction of proteins capable of modulating MHC Class I, wherein an alteration in activity of said enzyme is indicative of said drug's therapeutic potential in treating autoimmune disease or transplantation rejection.

21. The method of claim 26, wherein said enzyme is thioredoxin, superoxide dismutase or functional equivalents thereof.

22. An isolated nucleic acid comprising the Sox-4 nucleic acid sequence shown in Figure 20.

23. An isolated nucleic acid sequence comprising the Y-Box protein nucleic acid sequence shown in Figure 38.

24. A recombinant protein encoded by the nucleic acid sequence of claim 22.

25. A recombinant protein encoded by the nucleic acid sequence of claim 23.

26. An isolated and purified protein comprising the amino acid sequence shown in Figure 20 or Figure 38.

27. A recombinant expression vector comprising all or part of the nucleic acid sequence of claim 22 or 23.

28. A host organism transformed or transfected with the recombinant expression vector according to claim 26 in a manner to allow expression of said protein encoded by said recombinant expression vector.

29. Antibodies reactive with the protein according to claims 24 or 25 or portions thereof.

30. A method of preventing or treating transplantation rejection in a mammal comprising.

(a) isolating donor cells or tissue from a donor;

(b) genetically modifying said cells or tissue to express a nucleic acid sequence capable of MHC Class I suppression;

(c) transplanting said cells or tissue from step (b) into a recipient.

31. The method of claim 30 wherein said nucleic acid sequence encodes a Y-box protein or a Sox-4 protein or the functional equivalents thereof.

FIG. 1A

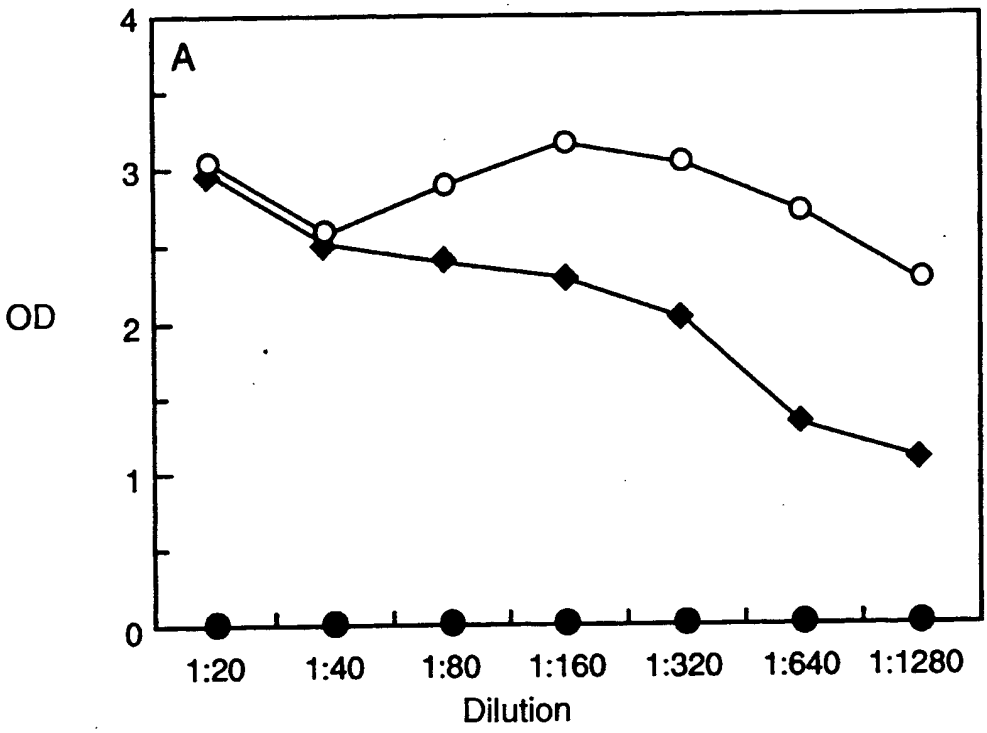

A
OD
4
3
2
1
0
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution

FIG. 1B

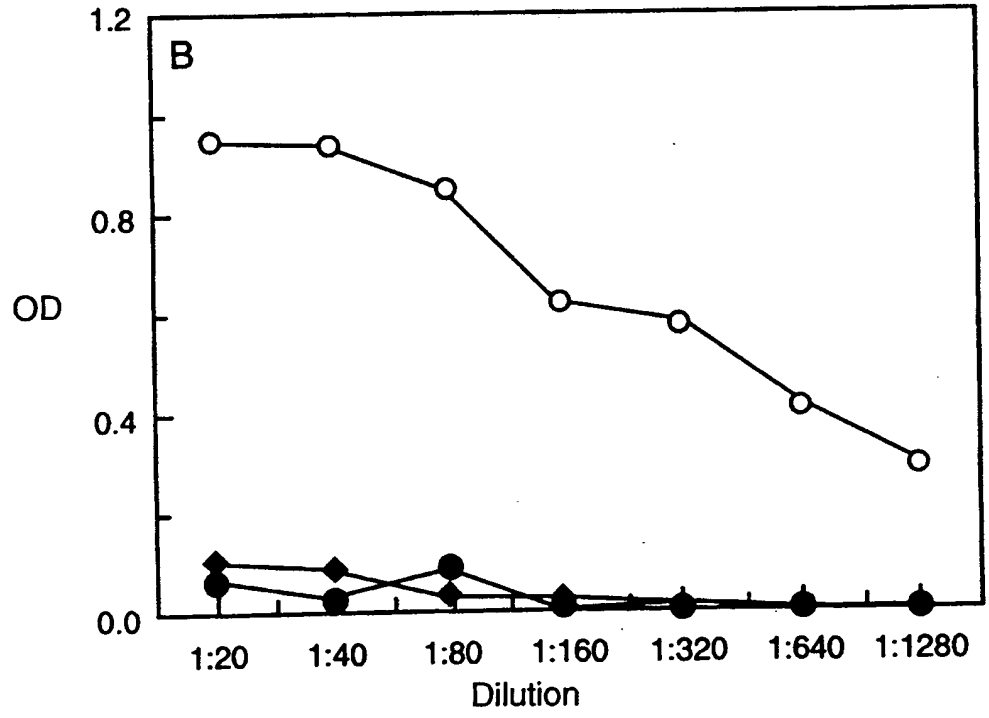

B
OD
1.2
0.8
0.4
0.0
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution

FIG. 1C

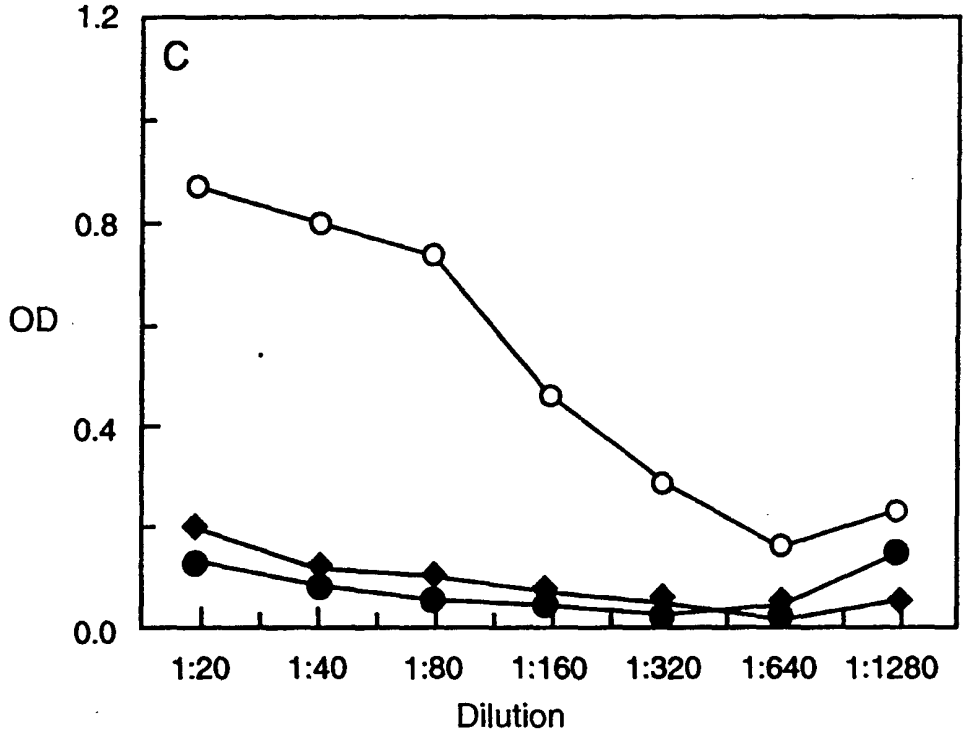

C
1.2
0.8
0.4
0.0
OD
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution

FIG. 1D

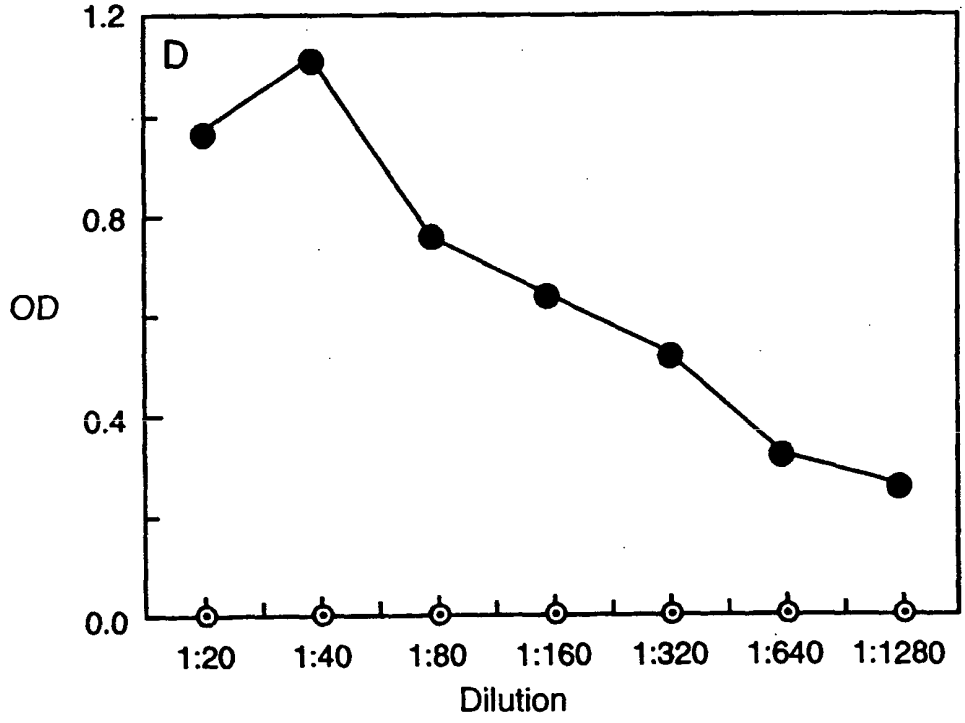

D
1.2
0.8
0.4
0.0
OD
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution

FIG. 2A

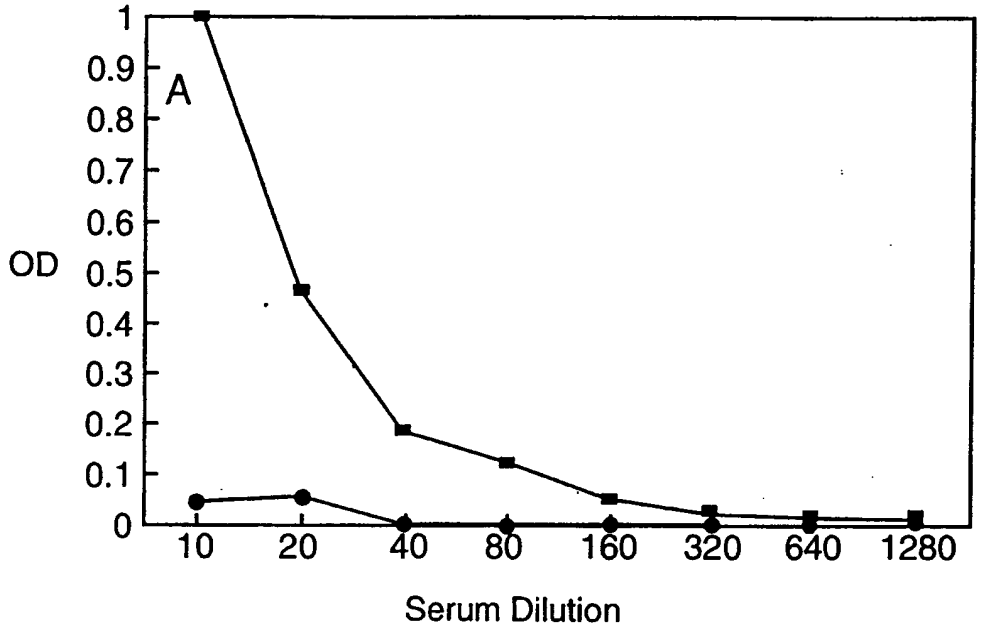

A
OD
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
40
80
160
320
640
1280
Serum Dilution

FIG. 2B

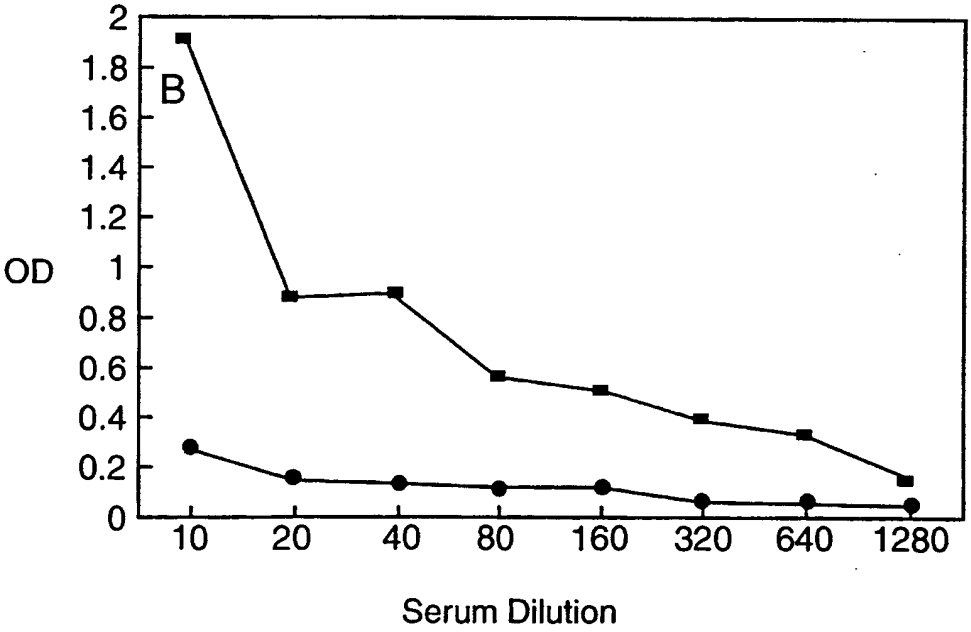

B
OD
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10
20
40
80
160
320
640
1280
Serum Dilution

FIG. 2C

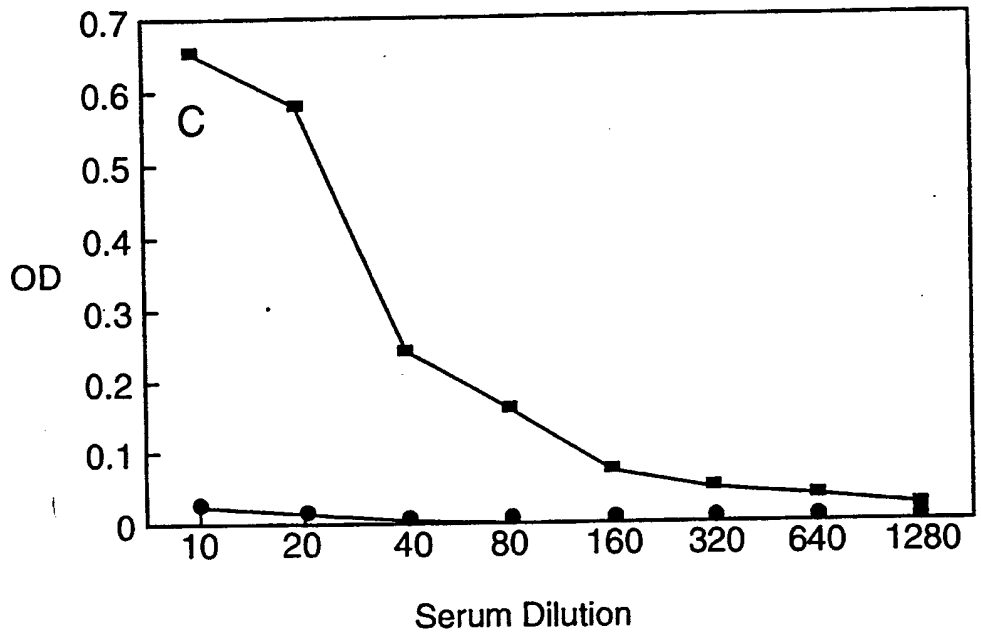

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD
C
10
20
40
80
160
320
640
1280
Serum Dilution

FIG. 2D

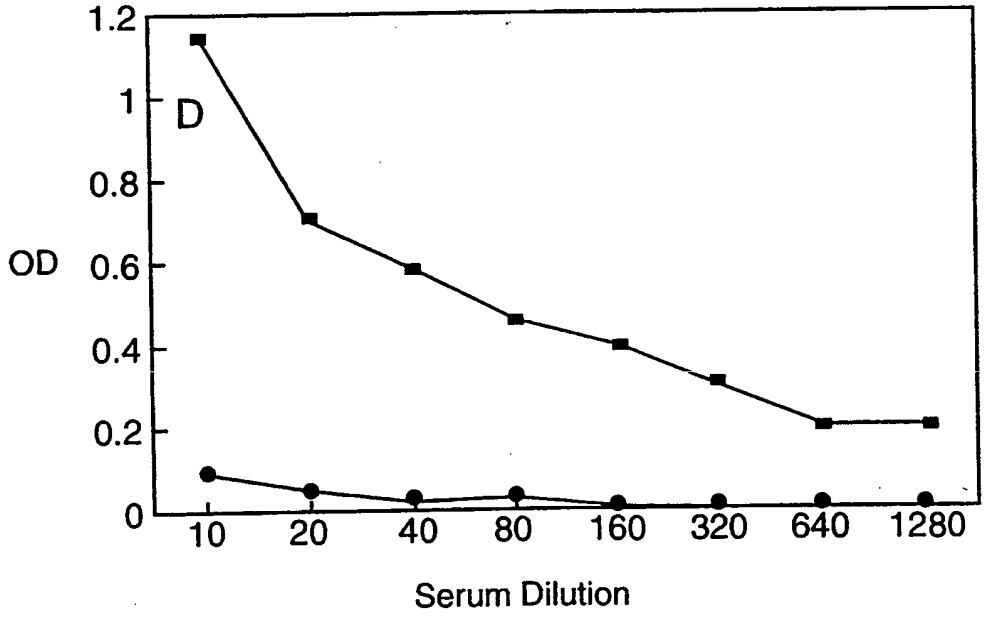

1.2
1
0.8
0.6
0.4
0.2
0
OD
D
10
20
40
80
160
320
640
1280
Serum Dilution

FIG. 3A

FIG. 3B

FIG. 4C

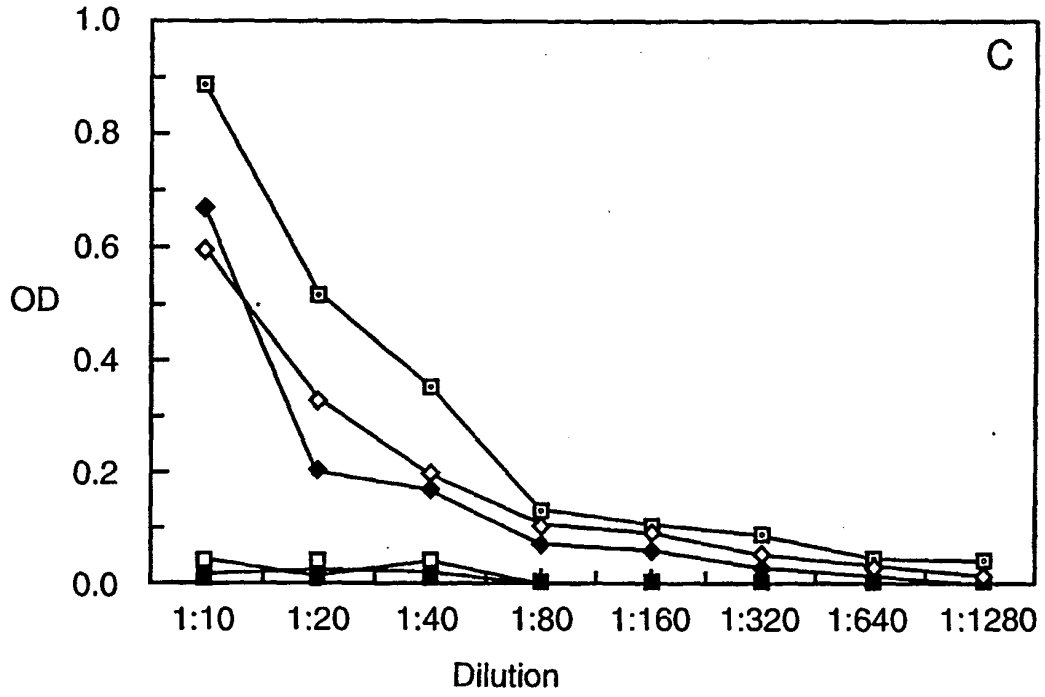

C
1.0
0.8
0.6
0.4
0.2
0.0
OD
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution

FIG. 4D

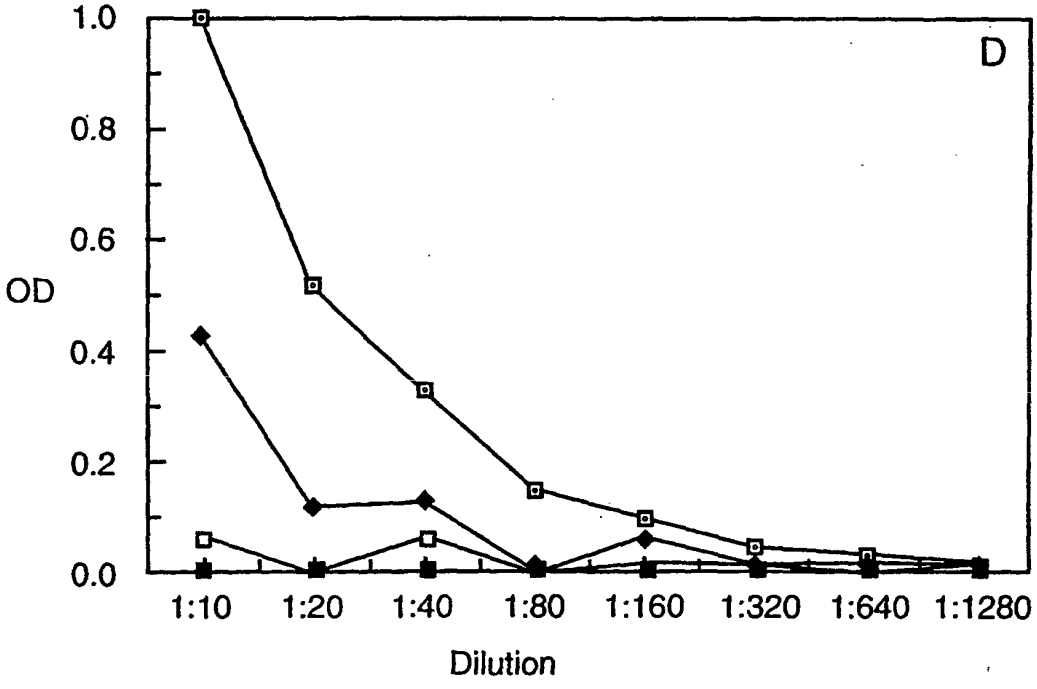

D
1.0
0.8
0.6
0.4
0.2
0.0
OD
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution

FIG. 4A

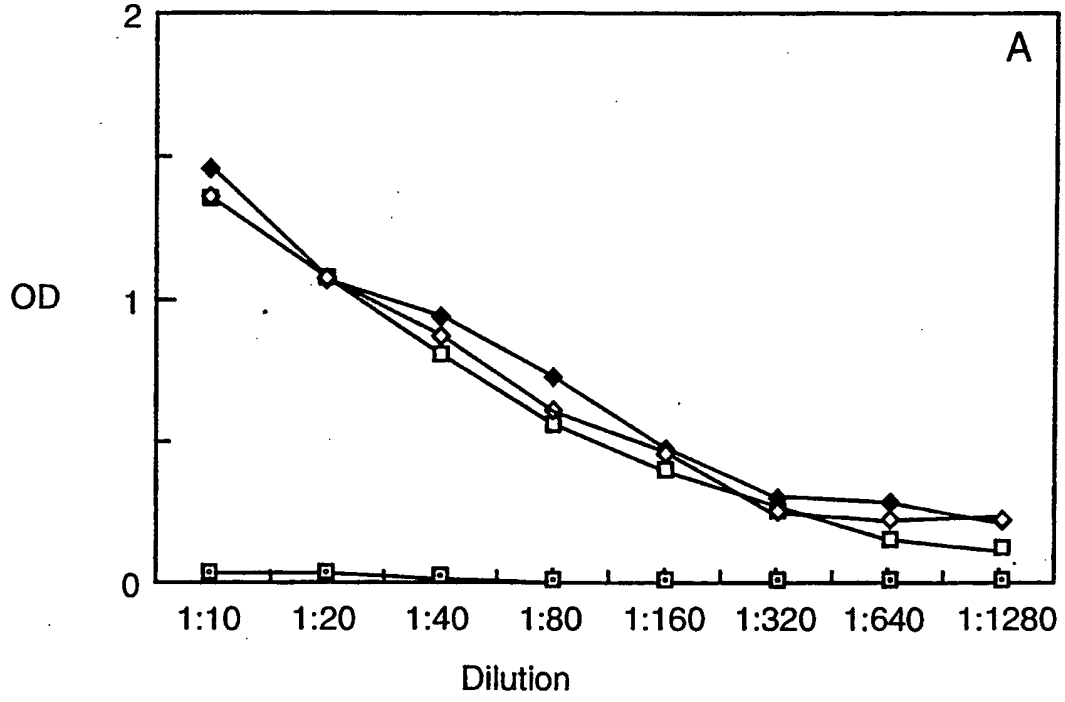

A
OD
2
1
0
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution

FIG. 4B

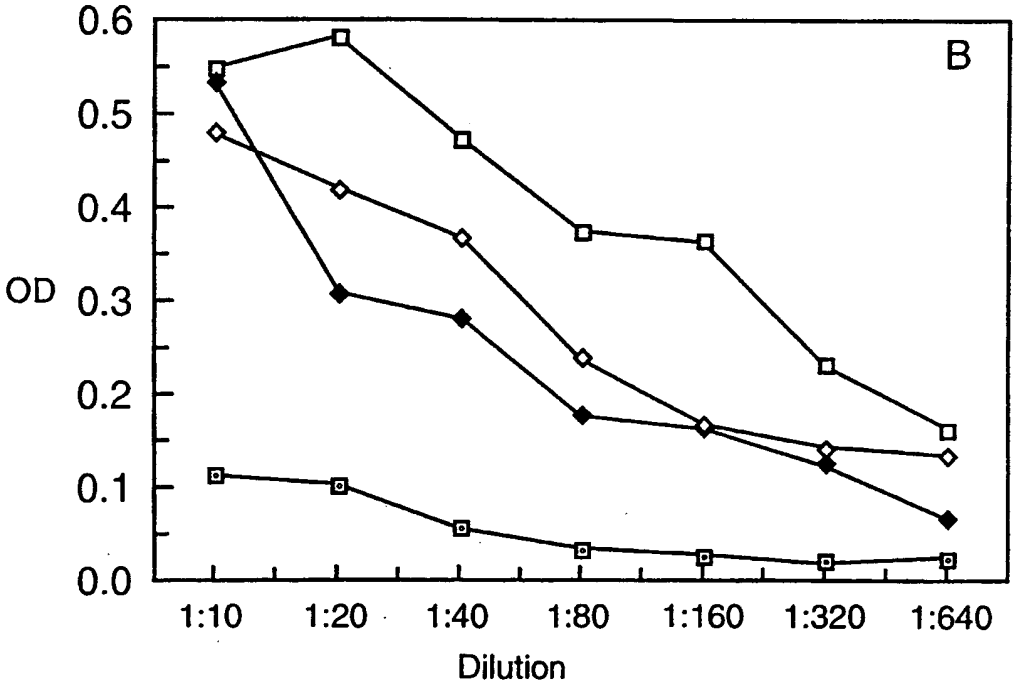

B
OD
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1:10
1:20
1:40
1:80
1:160
1:320
1:640
Dilution

FIG. 5

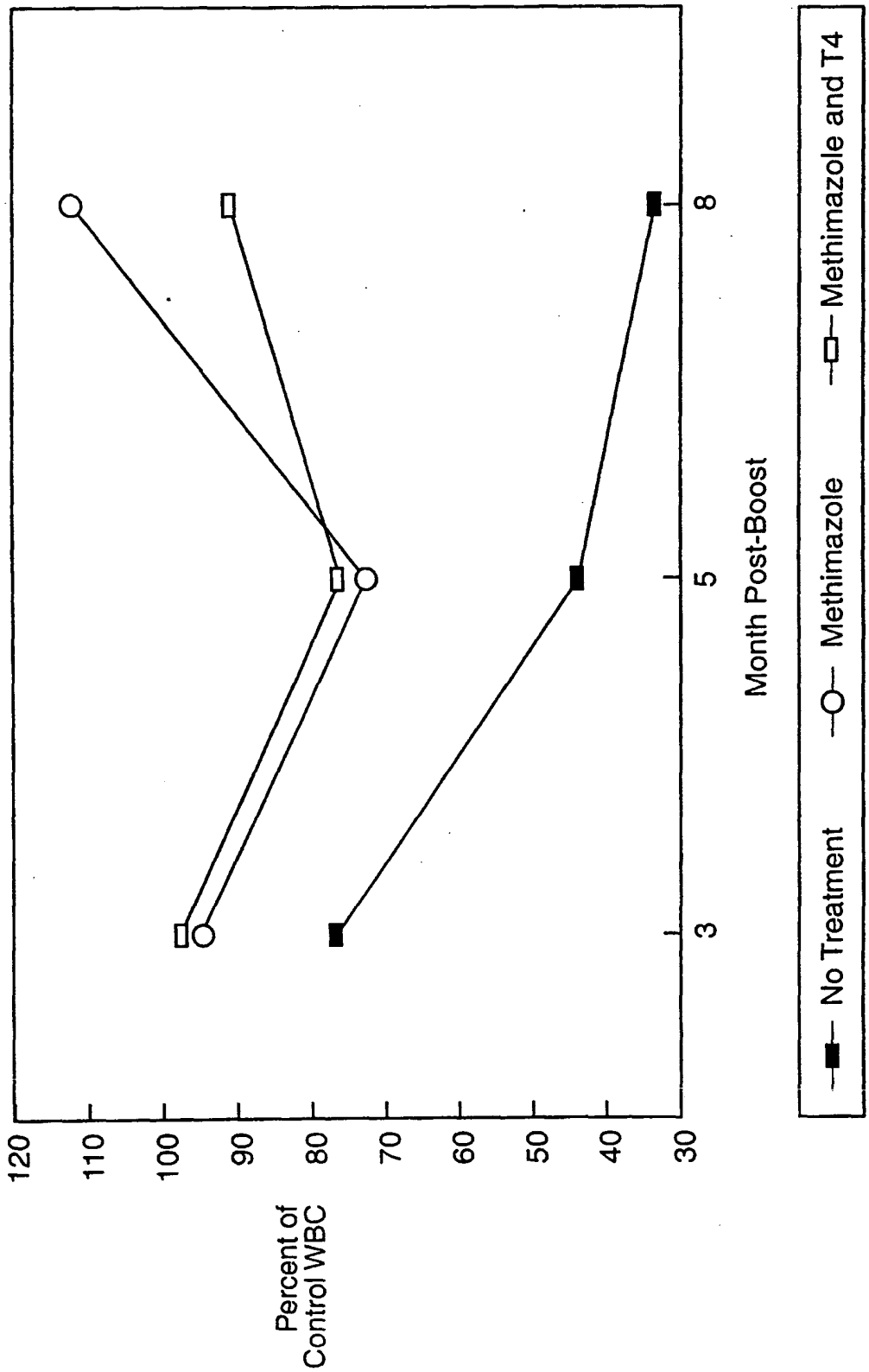

120
110
100
90
80
70
60
50
40
30
Percent of Control WBC
3
5
8
Month Post-Boost
No Treatment
Methimazole
Methimazole and T4

FIG. 6A

FIG. 6B

FIG. 7A

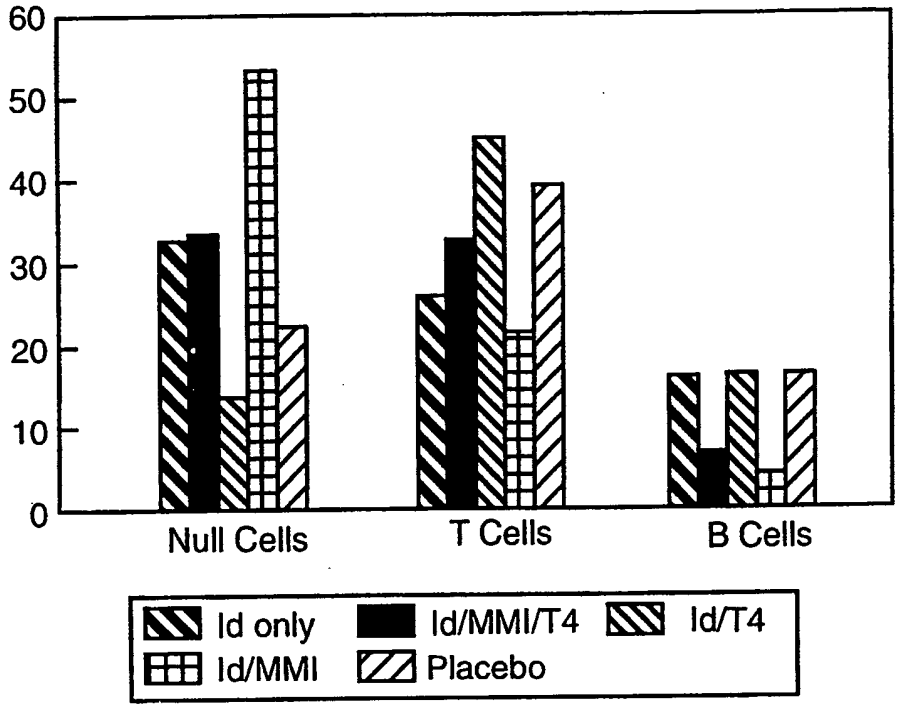

60
50
40
30
20
10
0
Null Cells
T Cells
B Cells
Id only
Id/MMI/T4
Id/T4
Id/MMI
Placebo

FIG. 7B

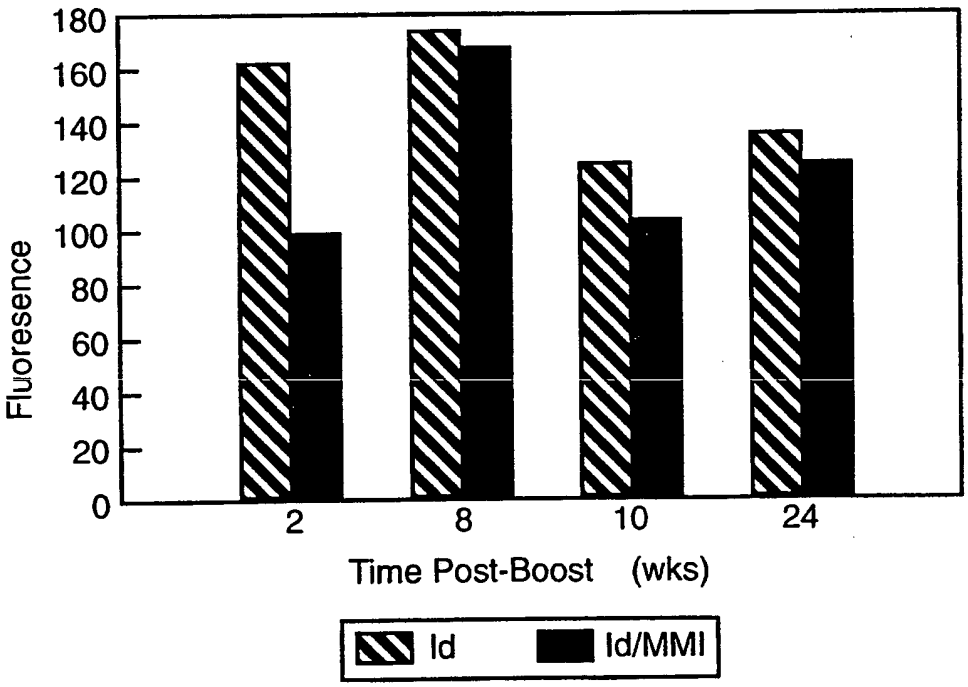

180
160
140
120
100
80
60
40
20
0
Fluoresence
2
8
10
24
Time Post-Boost (wks)
Id
Id/MMI

FIG. 7C

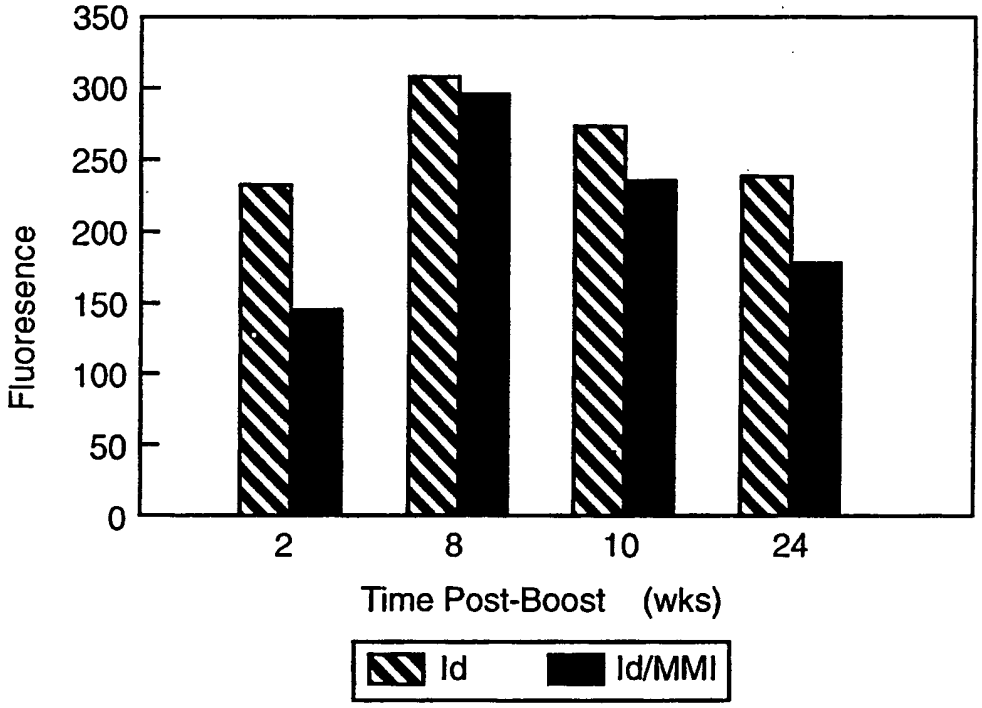

350
300
250
200
150
100
50
0
Fluoresence
2
8
10
24
Time Post-Boost (wks)
Id
Id/MMI

FIG. 7D

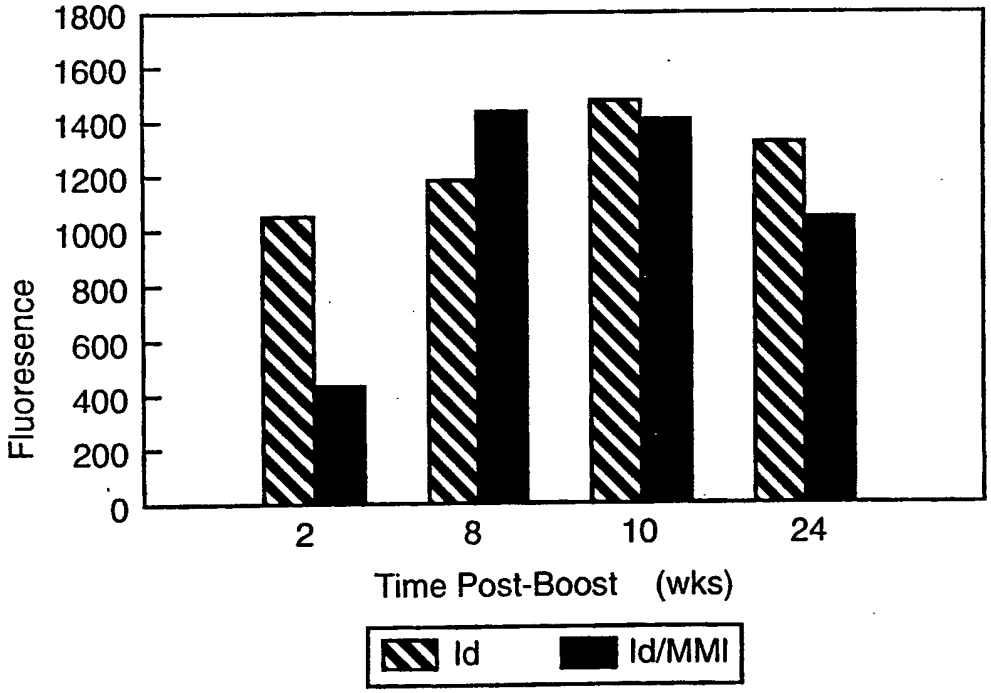

1800
1600
1400
1200
1000
800
600
400
200
0
Fluoresence
2
8
10
24
Time Post-Boost (wks)
Id
Id/MMI

FIG. 8

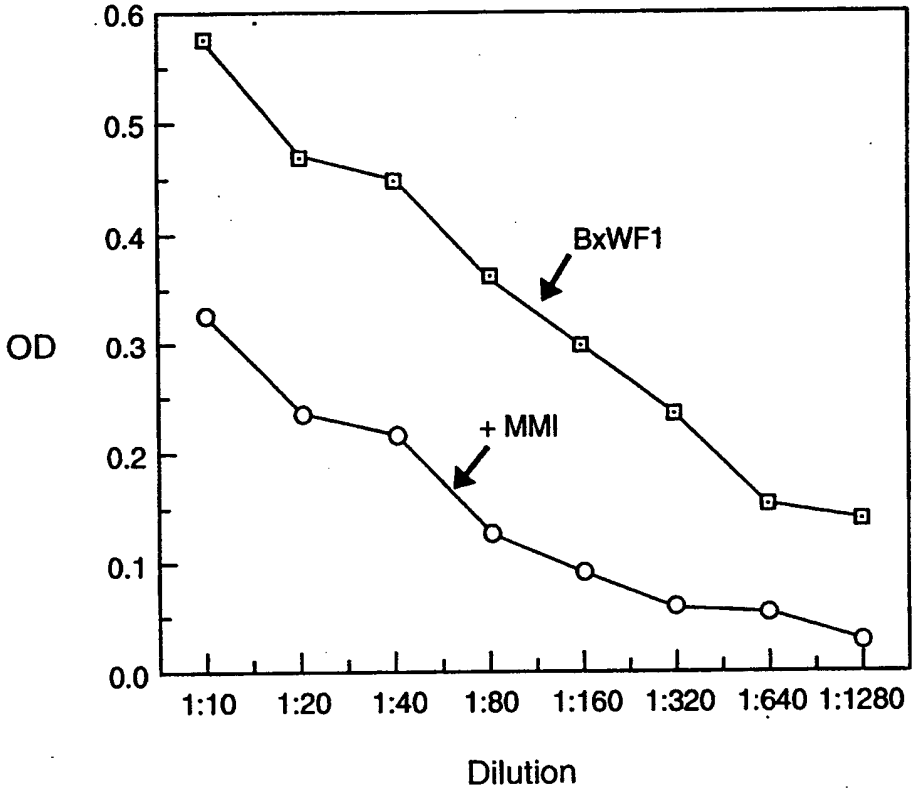

0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD
BxWF1
+ MMI
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
Dilution

FIG. 9A

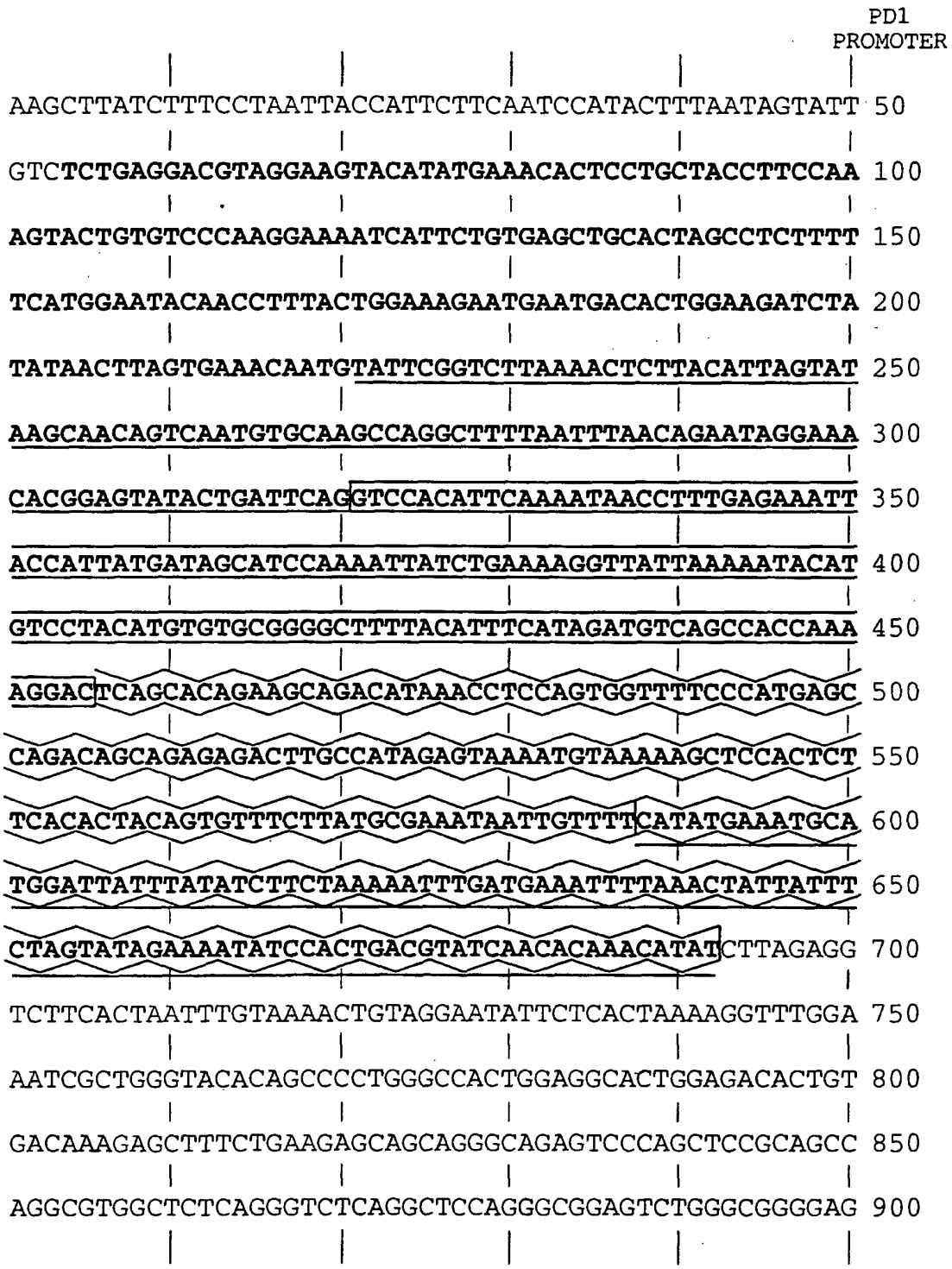

PD1
PROMOTER
AAGCTTATCTTTCCTAATTACCATTCTTCAATCCATACTTTAATAGTATT 50
GTCTCTGAGGACGTAGGAAGTACATATGAAACACTCCTGCTACCTTCCAA 100
AGTACTGTGTCCCAAGGAAAATCATTCTGTGAGCTGCACTAGCCTCTTTT 150
TCATGGAATACAACCTTTACTGGAAAGAATGAATGACACTGGAAGATCTA 200
TATAACTTAGTGAAACAATGTATTCGGTCTTAAAACTCTTACATTAGTAT 250
AAGCAACAGTCAATGTGCAAGCCAGGCTTTTAATTTAACAGAATAGGAAA 300
CACGGAGTATACTGATTCAGGTCCACATTCAAAATAACCTTTGAGAAATT 350
ACCATTATGATAGCATCCAAAATTATCTGAAAAGGTTATTAAAAATACAT 400
GTCCTACATGTGTGCGGGGCTTTTACATTTCATAGATGTCAGCCACCAAA 450
AGGACTCAGCACAGAAGCAGACATAAACCTCCAGTGGTTTTCCCATGAGC 500
CAGACAGCAGAGAGACTTGCCATAGAGTAAAATGTAAAAAGCTCCACTCT 550
TCACACTACAGTGTTTCTTATGCGAAATAATTGTTTTCATATGAAATGCA 600
TGGATTATTTATATCTTCTAAAAATTTGATGAAATTTTAAACTATTATTT 650
CTAGTATAGAAAATATCCACTGACGTATCAACACAAACATATCTTAGAGG 700
TCTTCACTAATTTGTAAAACTGTAGGAATATTCTCACTAAAAGGTTTGGA 750
AATCGCTGGGTACACAGCCCCTGGGCCACTGGAGGCACTGGAGACACTGT 800
GACAAAGAGCTTTCTGAAGAGCAGCAGGGCAGAGTCCCAGCTCCGCAGCC 850
AGGCGTGGCTCTCAGGGTCTCAGGCTCCAGGGCGGAGTCTGGGCGGGGAG 900

FIG. 9B

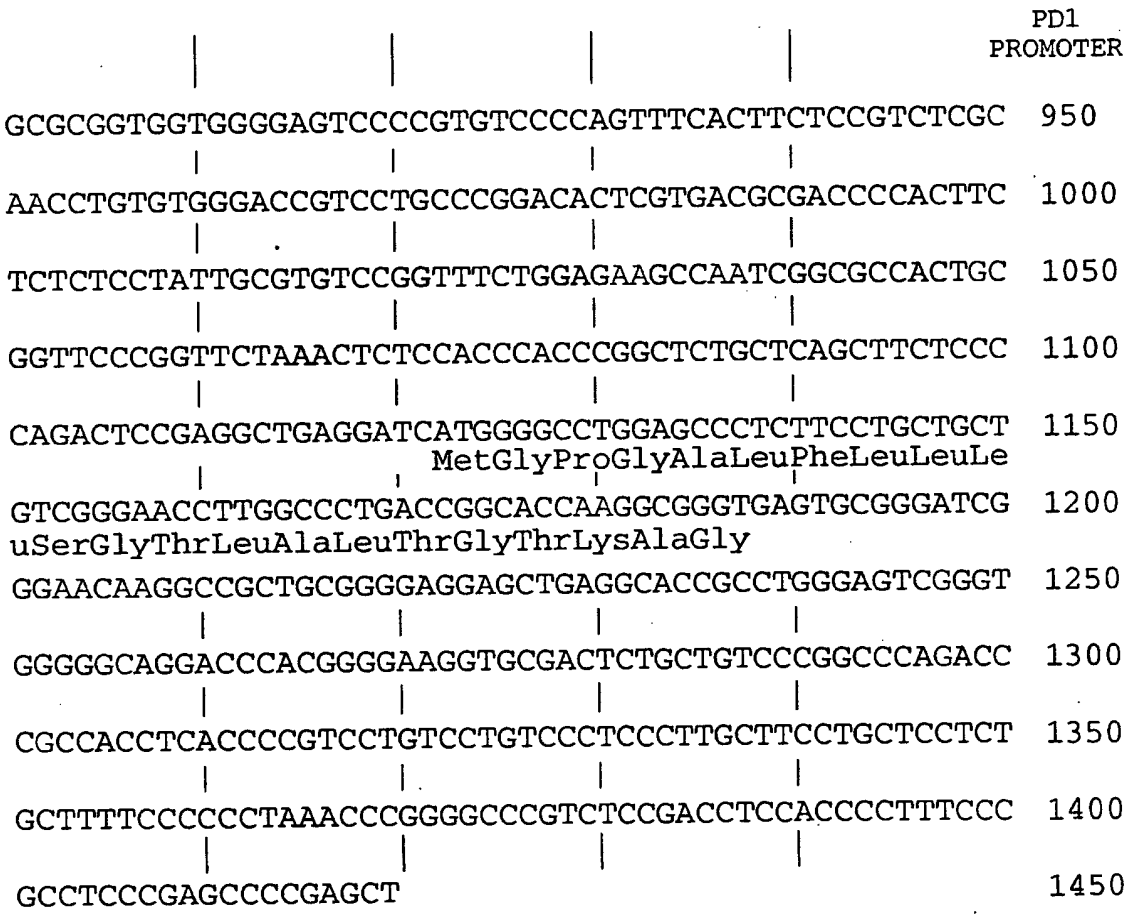

PD1
PROMOTER
GCGCGGTGGTGGGGAGTCCCCGTGTCCCCAGTTTCACTTCTCCGTCTCGC 950
AACCTGTGTGGGACCGTCCTGCCCGGACACTCGTGACGCGACCCCACTTC 1000
TCTCTCCTATTGCGTGTCCGGTTTCTGGAGAAGCCAATCGGCGCCACTGC 1050
GGTTCCCGGTTCTAAACTCTCCACCCACCCGGCTCTGCTCAGCTTCTCCC 1100
CAGACTCCGAGGCTGAGGATCATGGGGCCTGGAGCCCTCTTCCTGCTGCT 1150
MetGlyProGlyAlaLeuPheLeuLeLe
GTCGGGAACCTTGGCCCTGACCGGCACCAAGGCGGGTGAGTGCGGGATCG 1200
uSerGlyThrLeuAlaLeuThrGlyThrLysAlaGly
GGAACAAGGCCGCTGCGGGGAGGAGCTGAGGCACCGCCTGGGAGTCGGGT 1250
GGGGGCAGGACCCACGGGGAAGGTGCGACTCTGCTGTCCCGGCCCAGACC 1300
CGCCACCTCACCCCGTCCTGTCCTGTCCCTCCCTTGCTTCCTGCTCCTCT 1350
GCTTTTCCCCCCTAAACCCGGGGCCCGTCTCCGACCTCCACCCCTTTCCC 1400
GCCTCCCGAGCCCCGAGCT 1450

FIG. 10

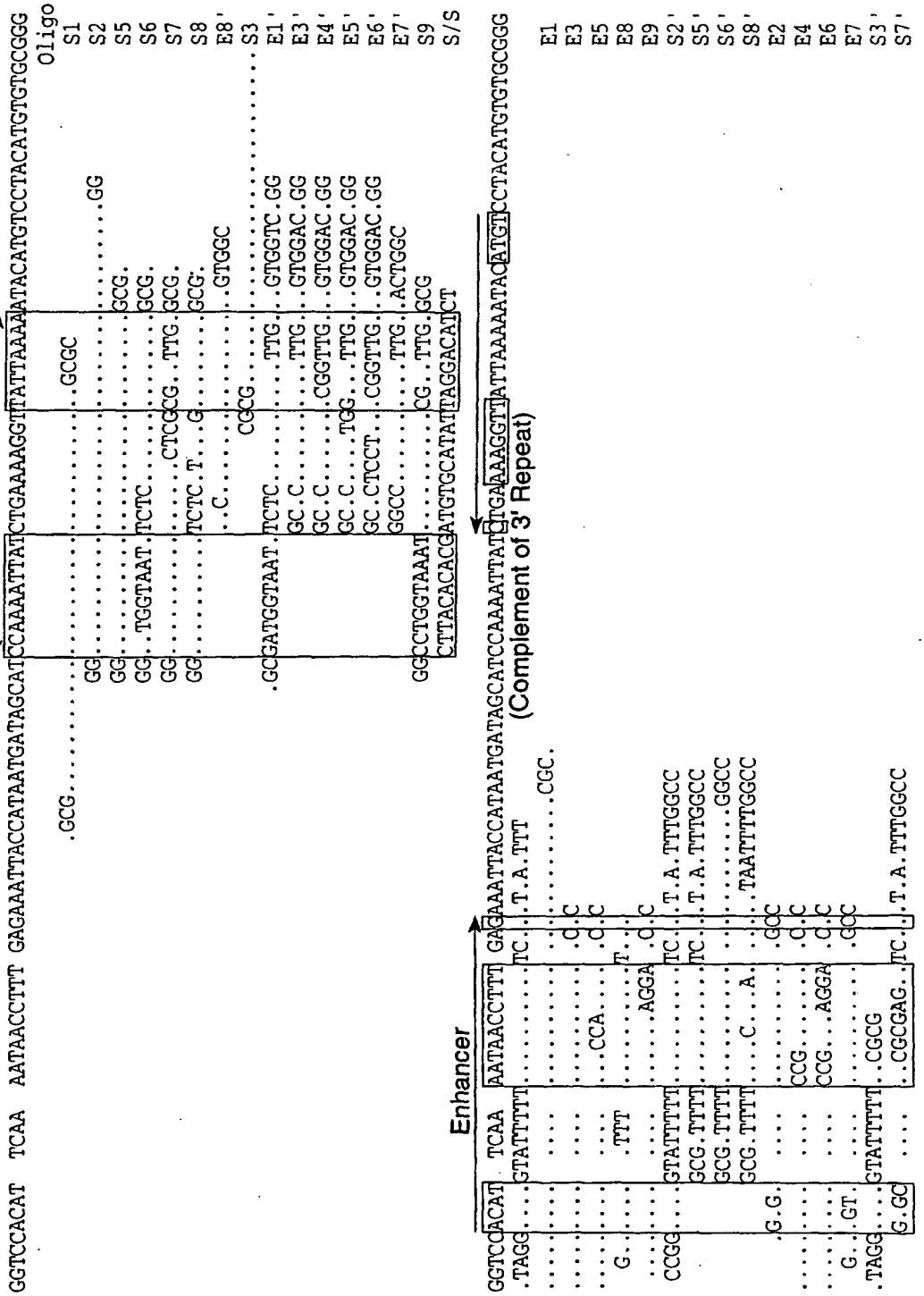

Silencer
TTF-2 Like
Enhancer
(Complement of 3' Repeat)
Oligo
S1
S2
S5
S6
S7
S8
E8'
S3
E1'
E3'
E4'
E5'
E6'
E7'
S9
S/S
E1
E3
E5
E8
E9
S2'
S5'
S6'
S8'
E2
E4
E6
E7
S3'
S7'

FIG. 11

```
105   -----CATATGAAATG--CATGGA---------TTATTTATATCTTCTAA   34
114   TGAAACAATGTATTCGGTCTAAAA-CTCTTACATTAGTATAAGCAACAGT   49
140   GTCCACATTCAAAATAACCTTTGAGAAATTACCATAATGATAGCATCCAA   50
          **..  .*..  .   *.....*         .**.*...*.*..*...
                                                    <----

105   AAATTT--------GATGAAATTTTAAACTATTATTTCTAGTATA--GAA   74
114   CAATGTGCAAGCCAGGCTTTTAATTTAACAGAA-----TAGGAAACACGG   94
140   -AATTATCTGAAAAGGTTATTAAAAATACATGTCCTACATGTGTGCGGGG   99
       ***..       *.  ........**....    ..*....    ..
      ------  TTF-2  ------------>

105   AATATCCA---CTGACGTATCAAC-ACAAACATATC   106
114   AGTATACTGAT--------TCAG-------------   109
140   CTTTTACATTTCATAGATGTCAGCCACCAAAAGGAC   135
       .*.* *.          ***.
```

FIG. 12D

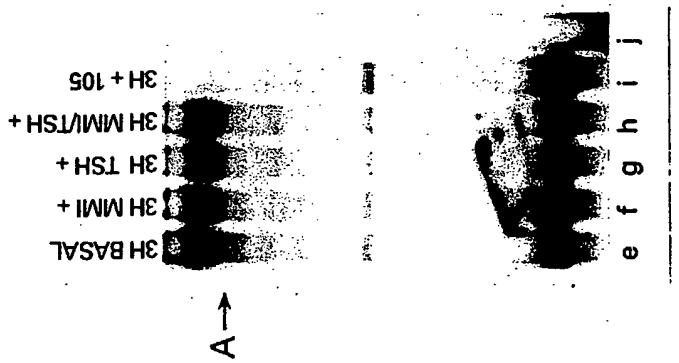


FIG. 12C

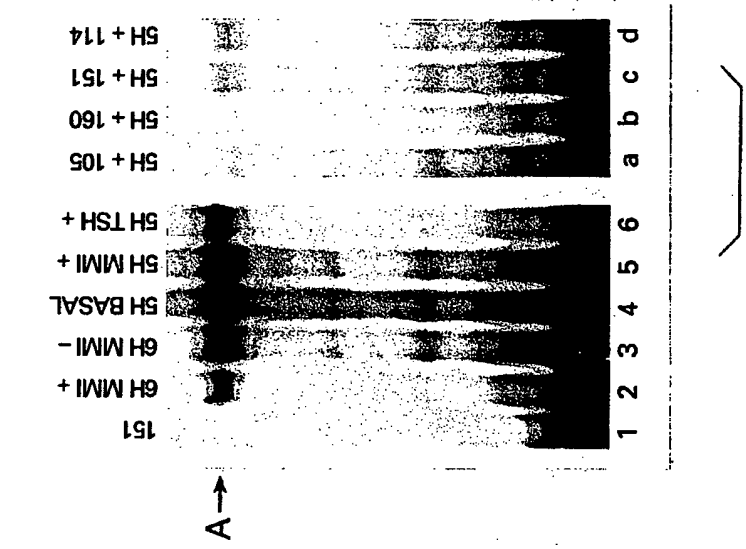


FIG. 12B

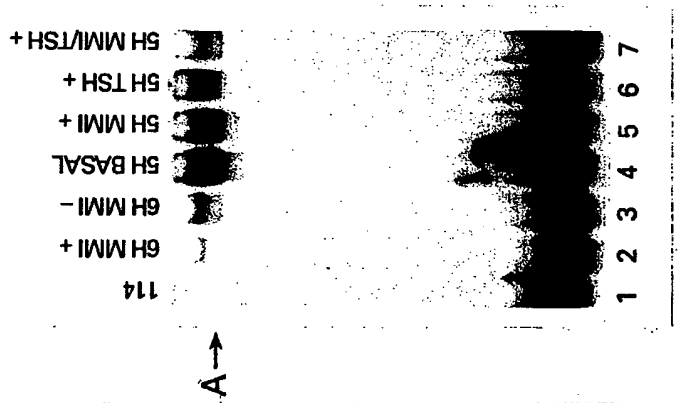


FIG. 12A

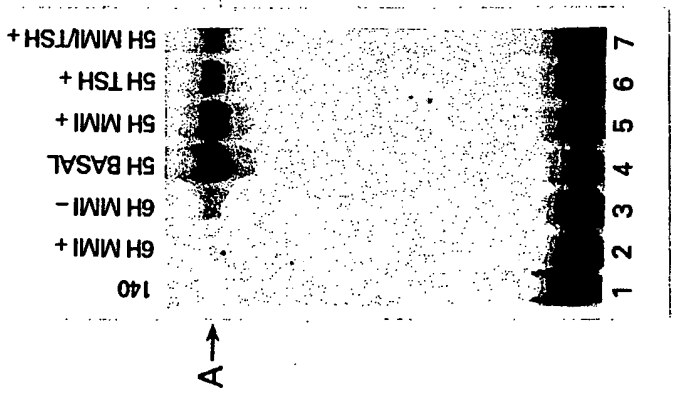


FIG. 13

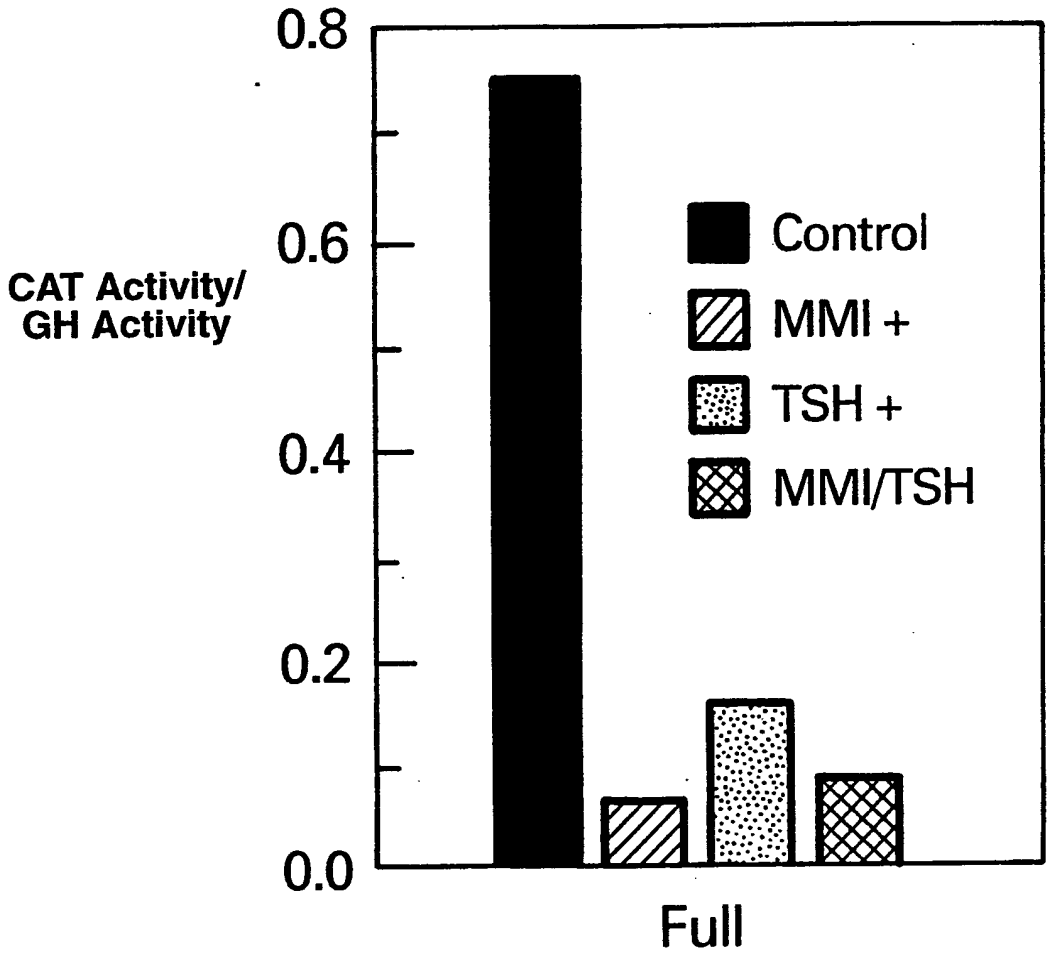

0.8
0.6
0.4
0.2
0.0
CAT Activity/
GH Activity
Control
MMI +
TSH +
MMI/TSH
Full

FIG. 14B

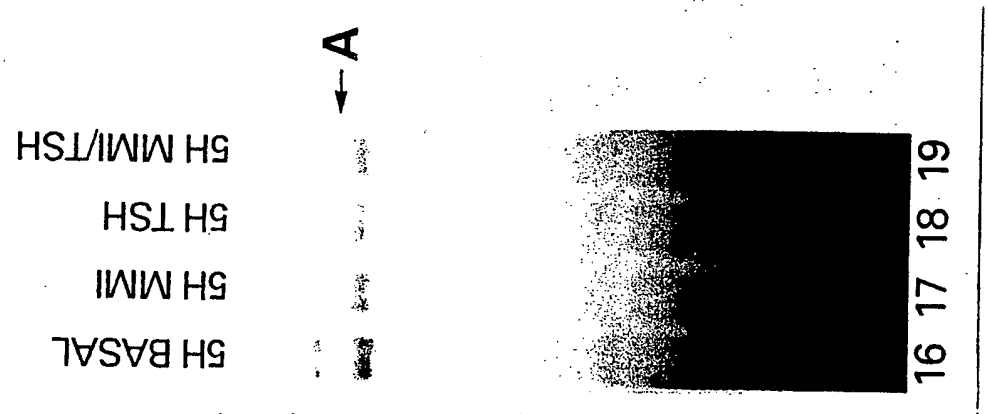


FIG. 14A

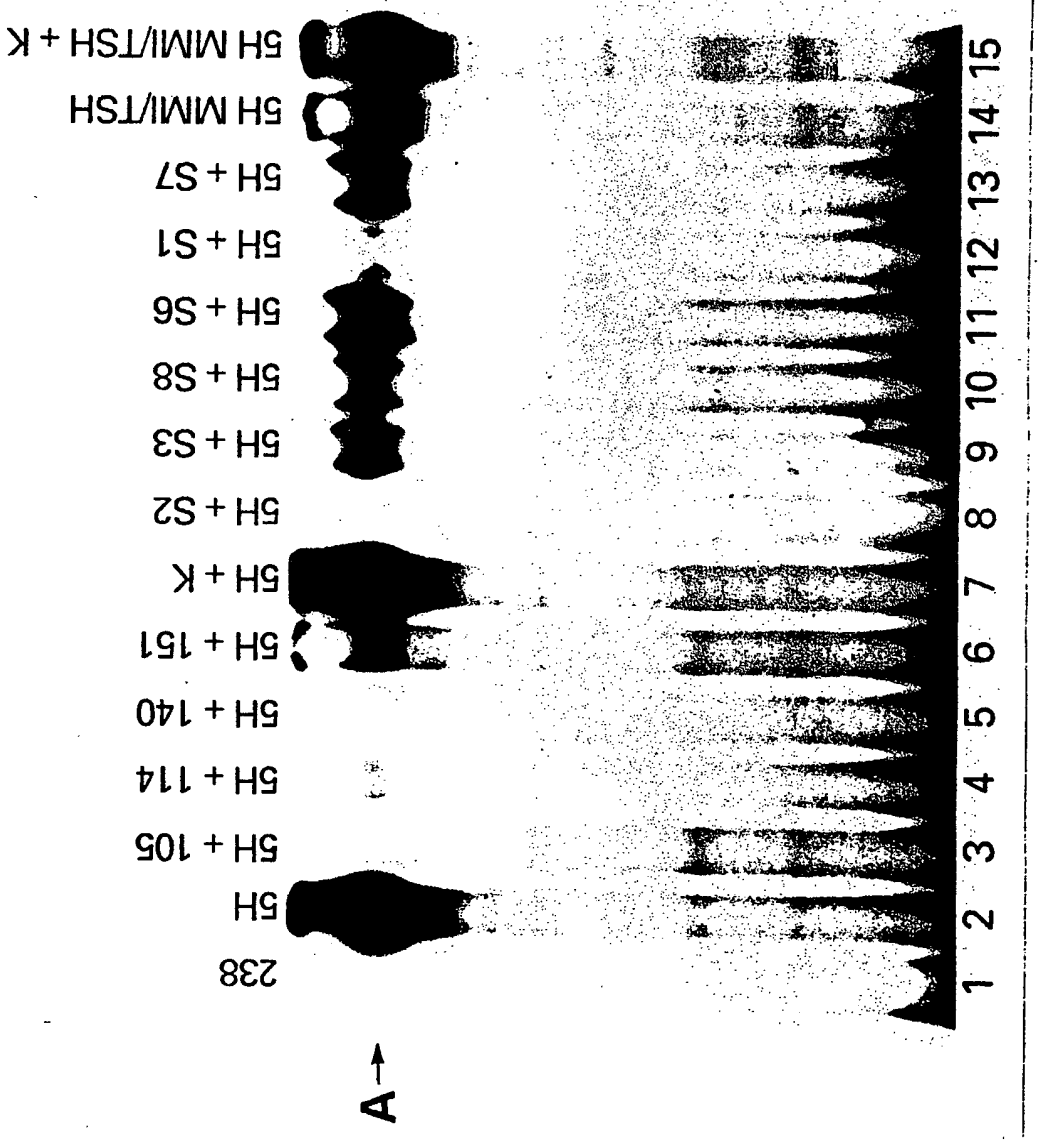


FIG. 15B

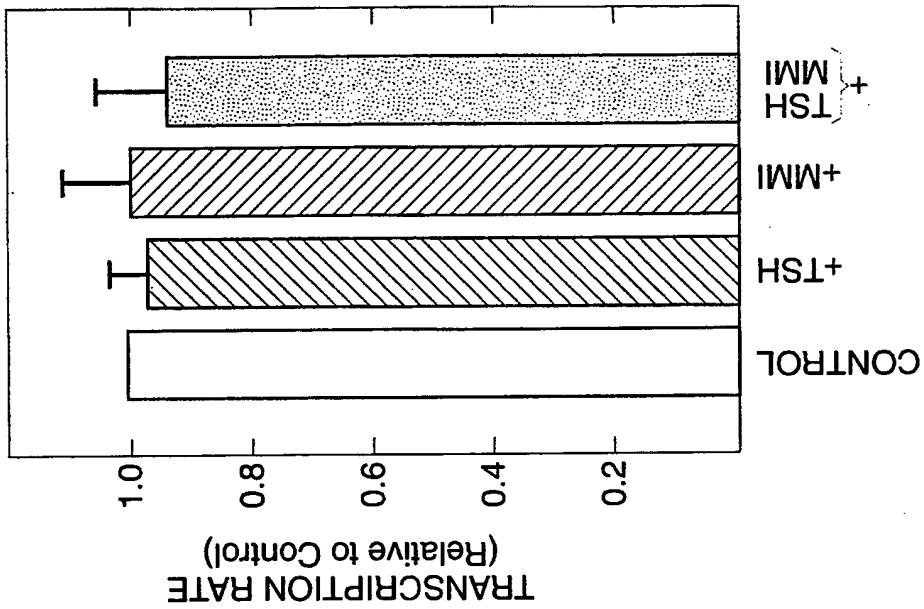


FIG. 15A

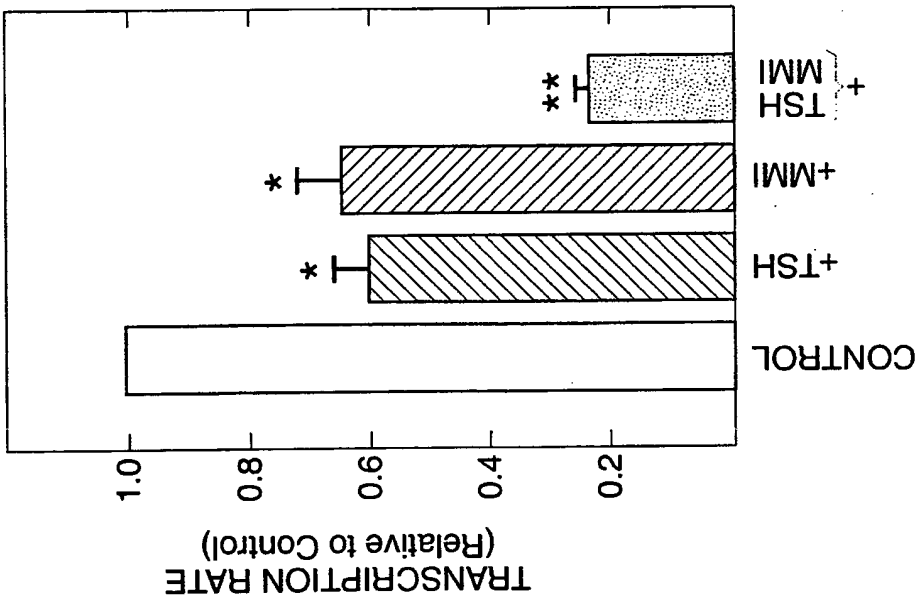


FIG. 16A

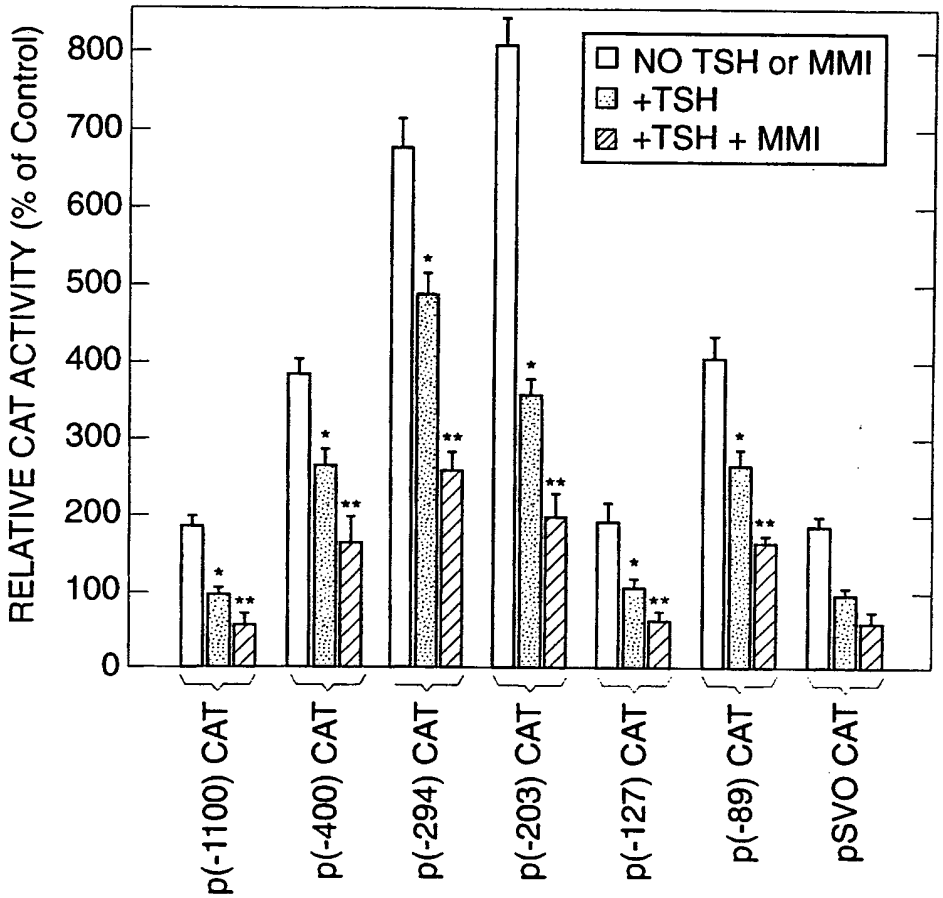

RELATIVE CAT ACTIVITY (% of Control)
0
100
200
300
400
500
600
700
800
NO TSH or MMI
+TSH
+TSH + MMI
p(-1100) CAT
p(-400) CAT
p(-294) CAT
p(-203) CAT
p(-127) CAT
p(-89) CAT
pSVO CAT

FIG. 16B

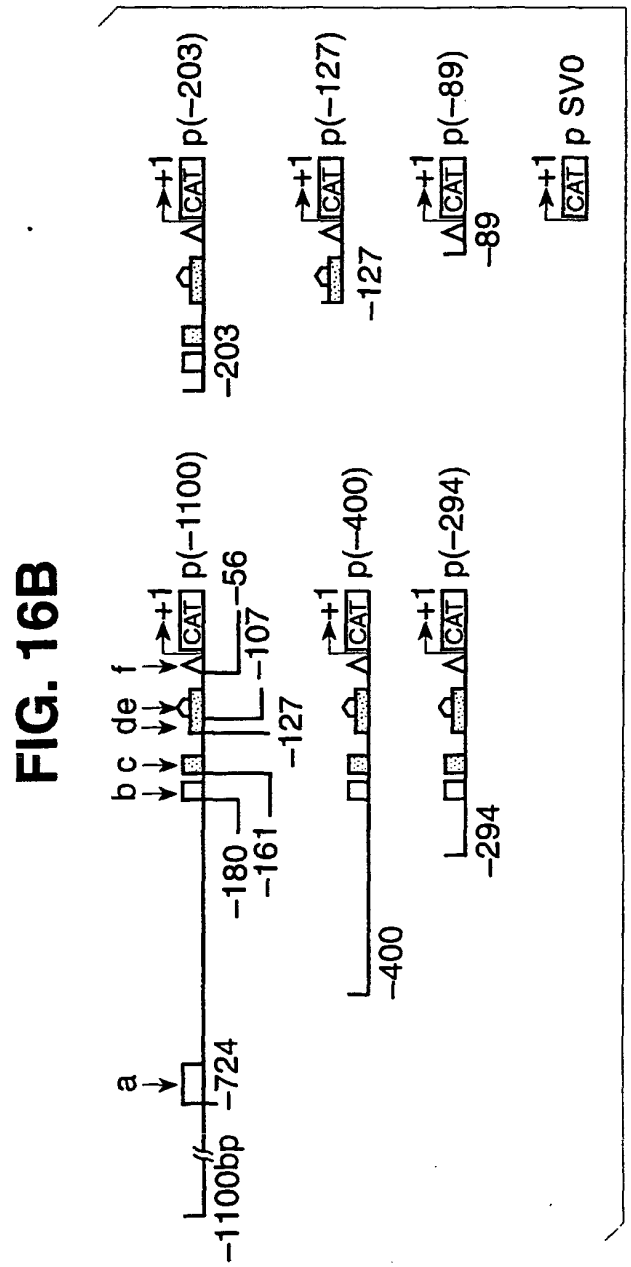

a
b c de f
−1100bp
−724
−180
−161
−127
−107
−56
−400
−294
−203
−127
−89
+1
CAT
p(−1100)
p(−400)
p(−294)
p(−203)
p(−127)
p(−89)
p SV0

FIG. 17B

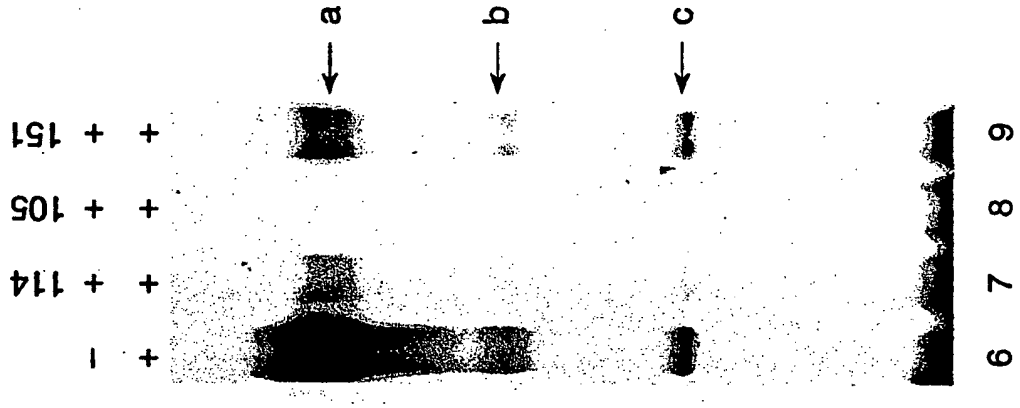
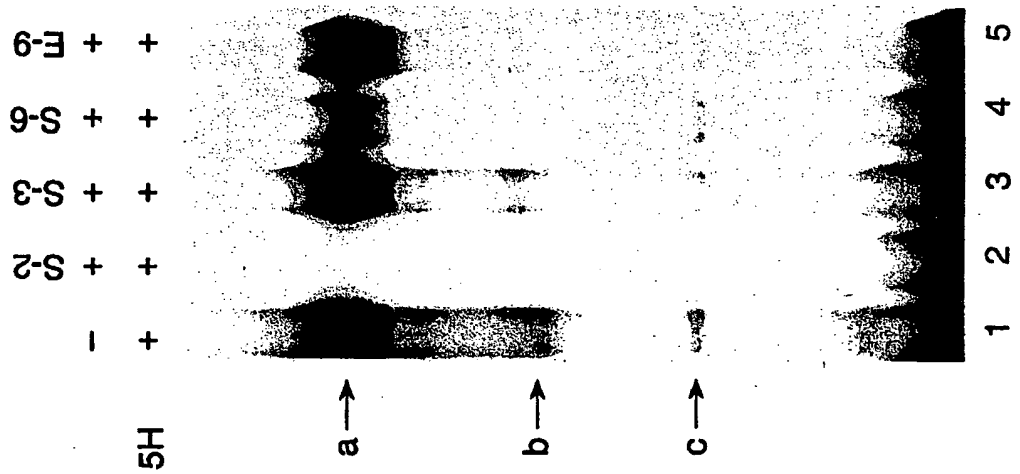


FIG. 17A



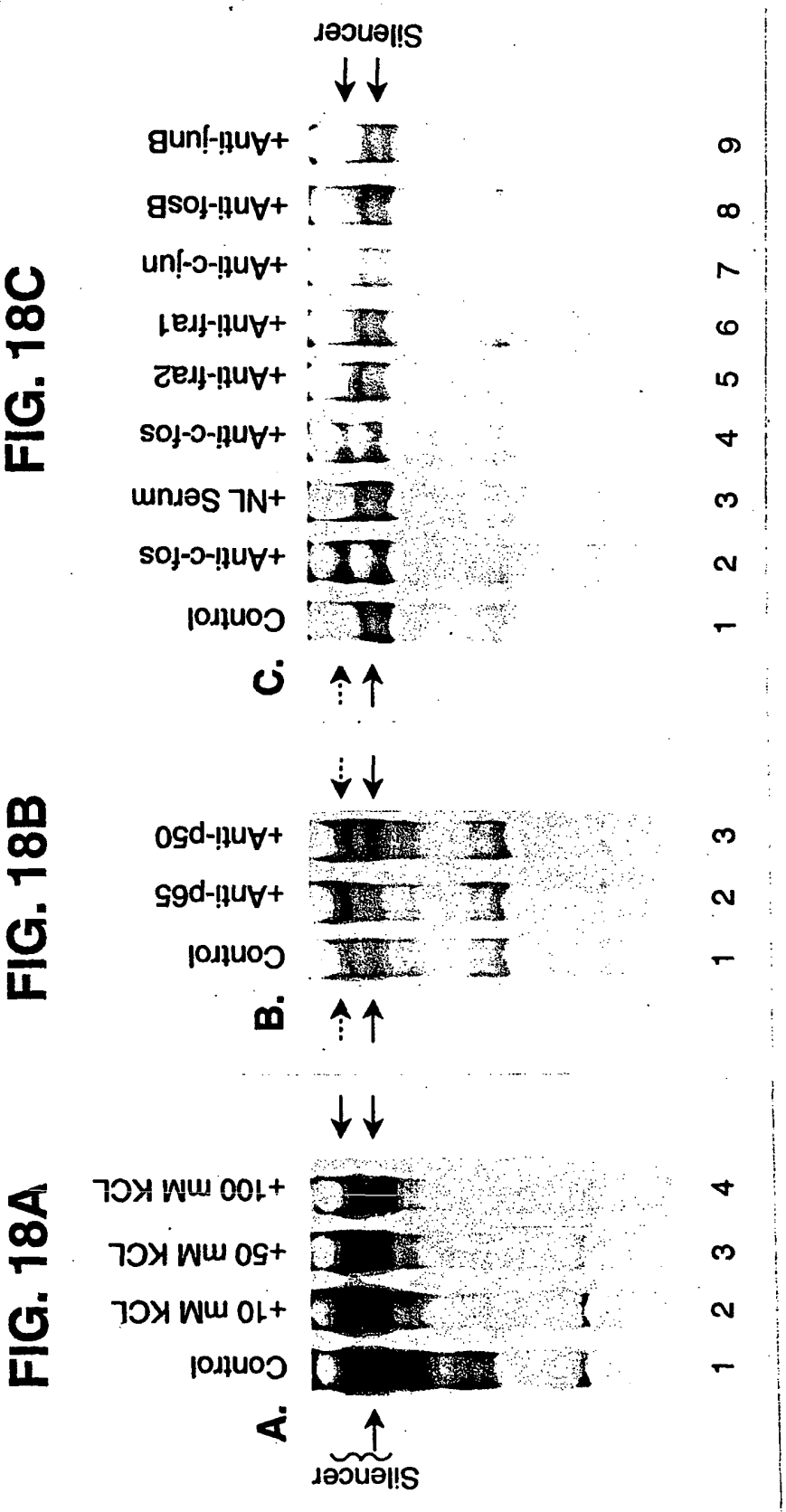


FIG. 18A
A.
Control
+10 mM KCL
+50 mM KCL
+100 mM KCL
1 2 3 4
Silencer
FIG. 18B
B.
Control
+Anti-p65
+Anti-p50
1 2 3
FIG. 18C
C.
Control
+Anti-c-fos
+NL Serum
+Anti-c-fos
+Anti-fra2
+Anti-fra1
+Anti-c-jun
+Anti-fosB
+Anti-junB
1 2 3 4 5 6 7 8 9
Silencer

FIG. 19C

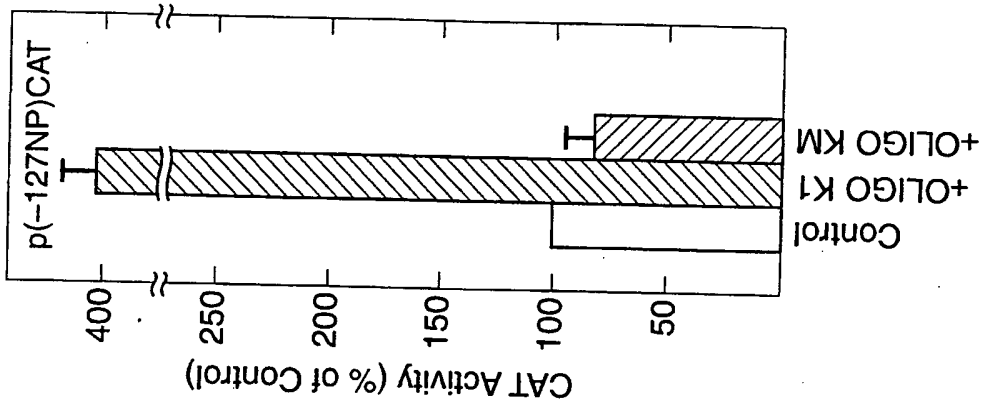


FIG. 19B

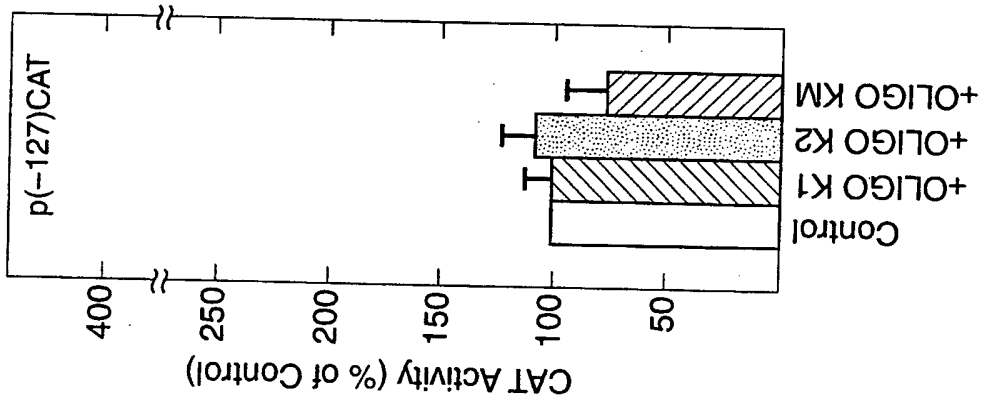


FIG. 19A

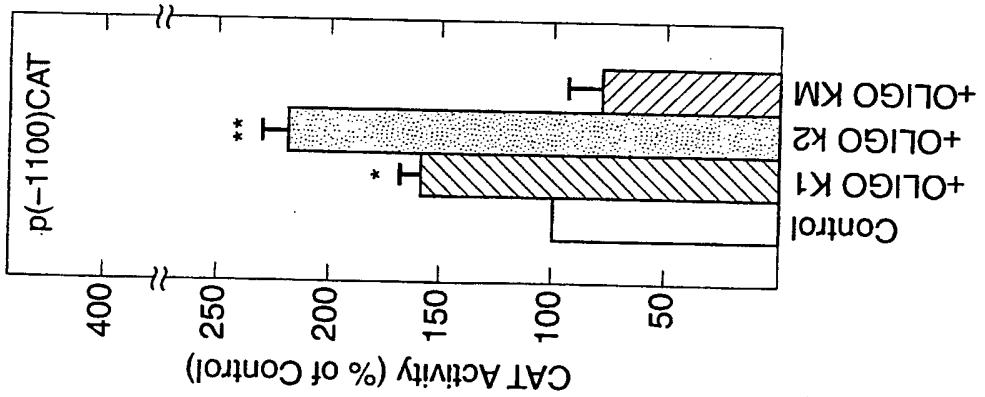


FIG. 20A

```
          10        20        30        40        50        60        70        80        90       100       110       120  NA/AA
aaagccgggccATGGTACAACAGACCAACAACGCCGAGAACACGGAGGCTTTGCTGGCTGGGGAGAGCTCAGACTCGGGCGGCGGCCTGGAGCTGGGCATCGCGTCCTCCCCGACGCCC  108
           MetValGlnGlnThrAsnAsnAlaGluAsnThrGluAlaLeuLeuAlaGlyGluSerSerAspSerGlyAlaGlyLeuGluLeuGlyIleAlaSerSerProThrPro   36

GGCTCCACGGCGTCCACGGGTGGCAAGGCGGACGACCCTAGCTGGTGCAAGACGCCCAGTGGCCACATCAAGCGGCCCATGAACGCCTTTATGGTGTGGTCGCAGATCGAGCGGCGCAAG  228
GlySerThrAlaSerThrGlyGlyLysAlaAspAspProSerTrpCysLysThrProSerGlyHisIleLysArgProMetAsnAlaPheMetValTrpSerGlnIleGluArgArgLys   76

ATCATGGAGCAGTCGCCCGACATGCACAACGCCGAGATCTCCAAGCGGCTGGGCAAACGCTGGAAGCTGCTCAAGGACAGCGACAAGATCCCGTTCATCCAGGAGGCGGAGCGGCTGCGC  348
IleMetGluGlnSerProAspMetHisAsnAlaGluIleSerLysArgLeuGlyLysArgTrpLysLeuLeuLysAspSerAspLysIleProPheIleGlnGluAlaGluArgLeuArg  116
                                                                                                       -

CTCAAGCACATGGCTGACTACCCTGACTACAAGTACCGGCCGCGAAAGAAGGTGAAGTCGGGCAACACGGGCGCGGGATCGGCGGCCACAGCCAAACCTGGGGAGAAGGGCGACAAGGTC  468
LeuLysHisMetAlaAspTyrProAspTyrLysTyrArgProArgLysLysValLysSerGlyAsnThrGlyAlaGlySerAlaAlaThrAlaLysProGlyGluLysGlyAspLysVal  156
                                                                 Ala - - - -     - -

GGCGGCGGCAGCGGCCACGCGGGAAGCGGCCACGCGGGGGGTGGCGCGGGCGGCAGCTCCAAGCCCGCGCCCAAGAAGAGCTGCGGCCCCAAGGTGGCGGGCAGCTCGGTCGGCAAGCCC  588
GlyGlyGlySerGlyHisAlaGlySerGlyHisAlaGlyGlyGlyAlaGlyGlySerSerLysProAlaProLysLysSerCysGlyProLysValAlaGlySerSerValGlyLysPro  196
Ala  Ser -     -     Ser -          -      Thr        Thr -                -         - -  -

CACGCCAAGTTCGTCCCGGCGGGCGGCGGTAAGGCGGCTGCATCGTTCTCTCCGGAGCAGGCCGCCCTGCTGCCCCTGGGGGAGCCCGCGGCCGTCTACAAGGTGCGGACTCCCAGCGCG  708
HisAlaLysPheValProAlaGlyGlyGlyLysAlaAlaAlaSerPheSerProGluGlnAlaAlaLeuLeuProLeuGlyGluProAlaAlaValTyrLysValArgThrProSerAla  236
         Leu - - -       Ser                  - -                          - - Thr - -      -                   GlnLeu
```

FIG. 20B

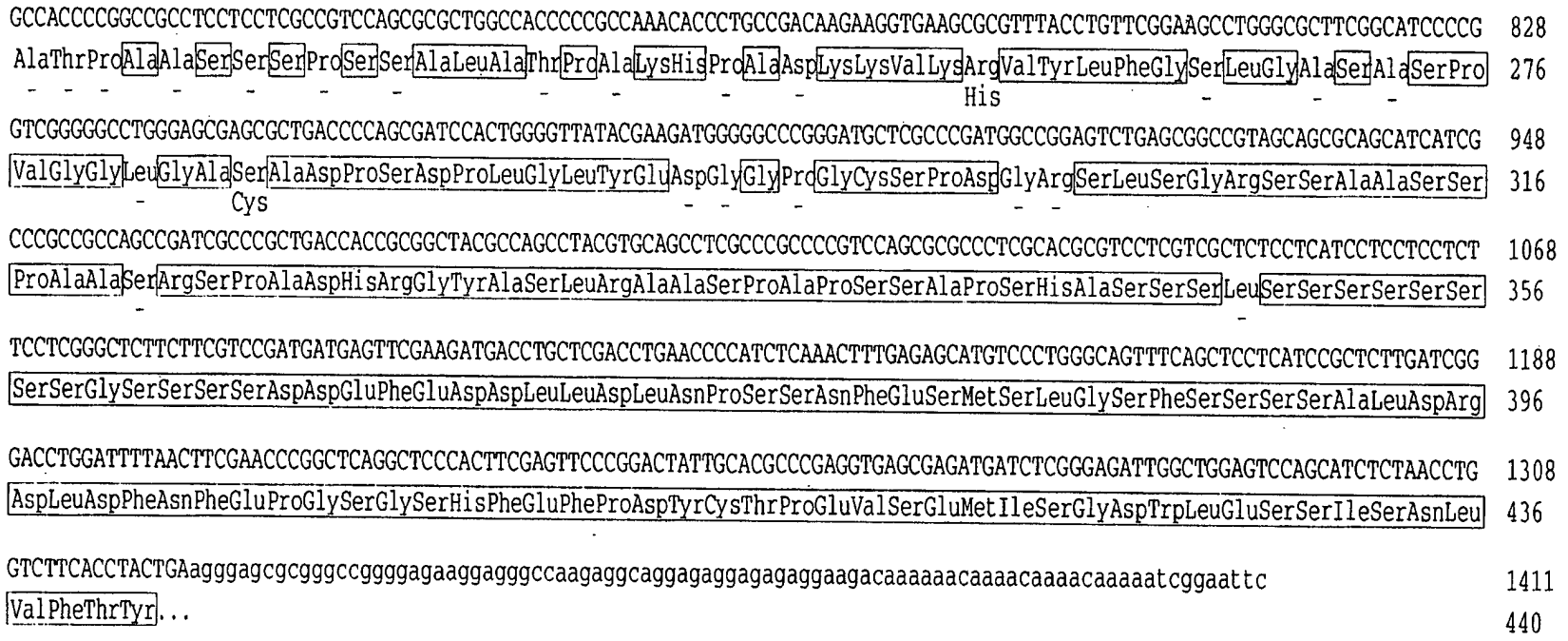

GCCACCCCGGCCGCCTCCTCCTCGCCGTCCAGCGCGCTGGCCACCCCCGCCAAACACCCTGCCGACAAGAAGGTGAAGCGCGTTTACCTGTTCGGAAGCCTGGGCGCTTCGGCATCCCCG 828
AlaThrProAlaAlaSerSerSerProSerSerAlaLeuAlaThrProAlaLysHisProAlaAspLysLysValLysArgValTyrLeuPheGlySerLeuGlyAlaSerAlaSerPro 276
His
GTCGGGGGCCTGGGAGCGAGCGCTGACCCCAGCGATCCACTGGGGTTATACGAAGATGGGGGCCCGGGATGCTCGCCCGATGGCCGGAGTCTGAGCGGCCGTAGCAGCGCAGCATCATCG 948
ValGlyGlyLeuGlyAlaSerAlaAspProSerAspProLeuGlyLeuTyrGluAspGlyGlyProGlyCysSerProAspGlyArgSerLeuSerGlyArgSerSerAlaAlaSerSer 316
Cys
CCCGCCGCCAGCCGATCGCCCGCTGACCACCGCGGCTACGCCAGCCTACGTGCAGCCTCGCCCGCCCCGTCCAGCGCGCCCTCGCACGCGTCCTCGTCGCTCTCCTCATCCTCCTCCTCT 1068
ProAlaAlaSerArgSerProAlaAspHisArgGlyTyrAlaSerLeuArgAlaAlaSerProAlaProSerSerAlaProSerHisAlaSerSerSerLeuSerSerSerSerSerSer 356
TCCTCGGGCTCTTCTTCGTCCGATGATGAGTTCGAAGATGACCTGCTCGACCTGAACCCCATCTCAAACTTTGAGAGCATGTCCCTGGGCAGTTTCAGCTCCTCATCCGCTCTTGATCGG 1188
SerSerGlySerSerSerSerAspAspGluPheGluAspAspLeuLeuAspLeuAsnProSerSerAsnPheGluSerMetSerLeuGlySerPheSerSerSerSerAlaLeuAspArg 396
GACCTGGATTTTAACTTCGAACCCGGCTCAGGCTCCCACTTCGAGTTCCCGGACTATTGCACGCCCGAGGTGAGCGAGATGATCTCGGGAGATTGGCTGGAGTCCAGCATCTCTAACCTG 1308
AspLeuAspPheAsnPheGluProGlySerGlySerHisPheGluPheProAspTyrCysThrProGluValSerGluMetIleSerGlyAspTrpLeuGluSerSerIleSerAsnLeu 436
GTCTTCACCTACTGAagggagcgcgggccggggagaaggagggccaagaggcaggagaggagagaggaagacaaaaaacaaaacaaaacaaaaatcggaattc 1411
ValPheThrTyr... 440

FIG. 21

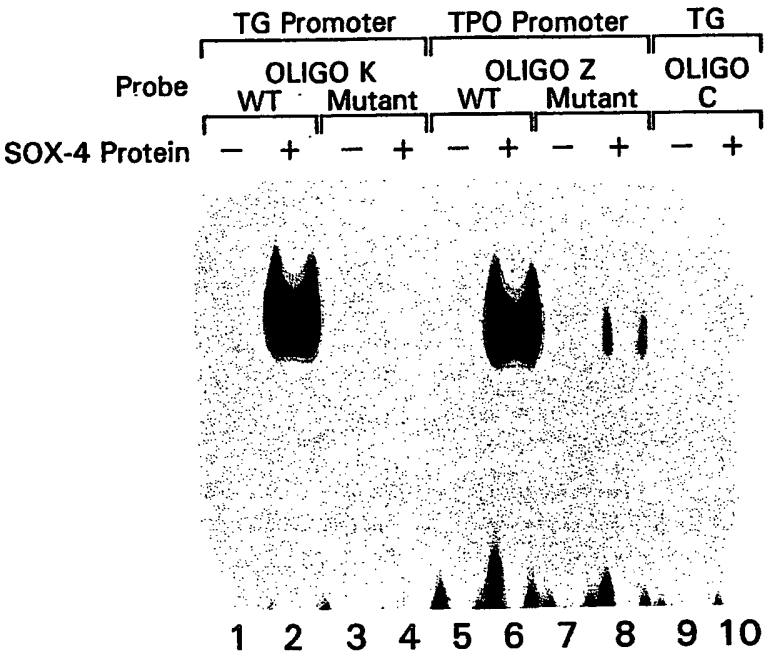

TG Promoter
TPO Promoter
TG
Probe
OLIGO K
WT
Mutant
OLIGO Z
WT
Mutant
OLIGO C
SOX-4 Protein
– + – + – + – + – +
1 2 3 4 5 6 7 8 9 10

FIG. 22A

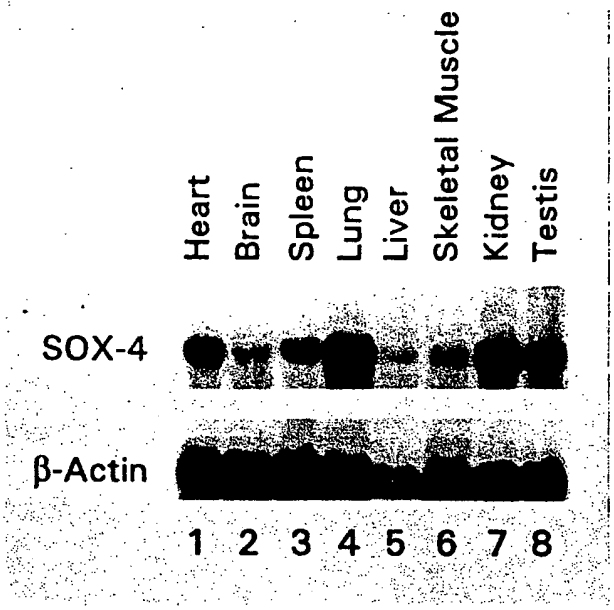


FIG. 22B

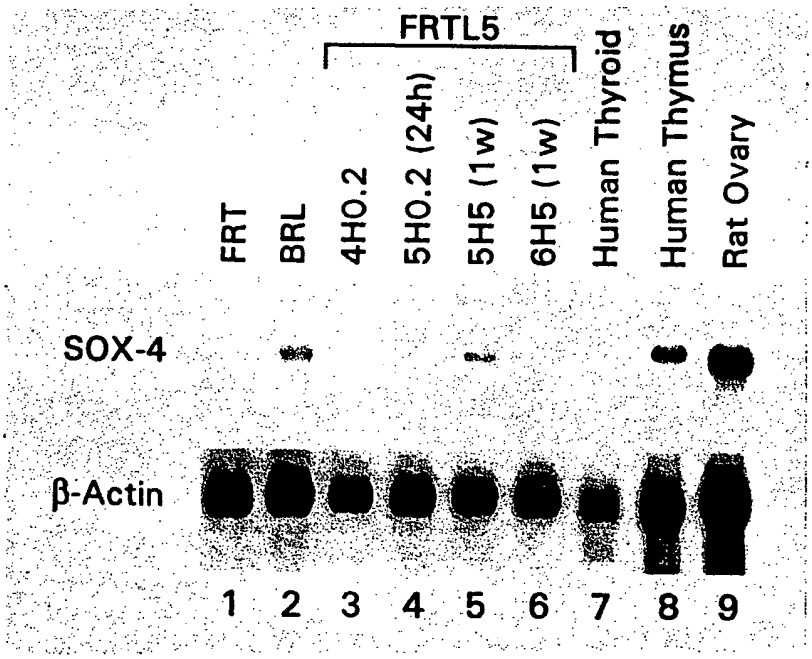


FIG. 23

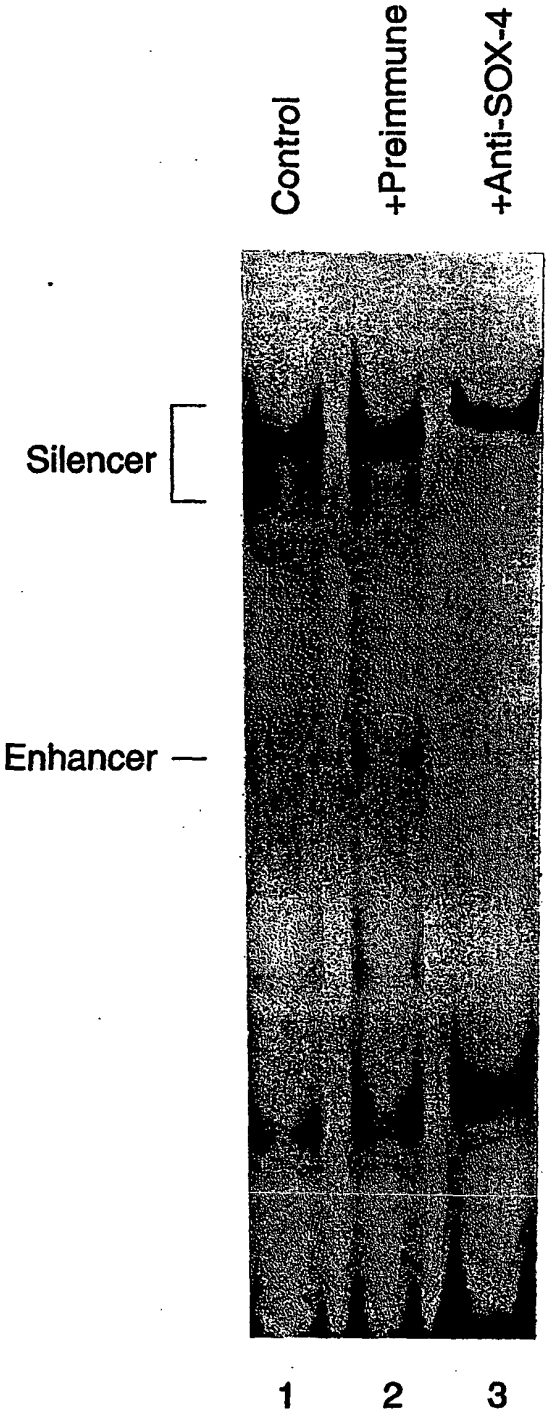


FIG. 24A

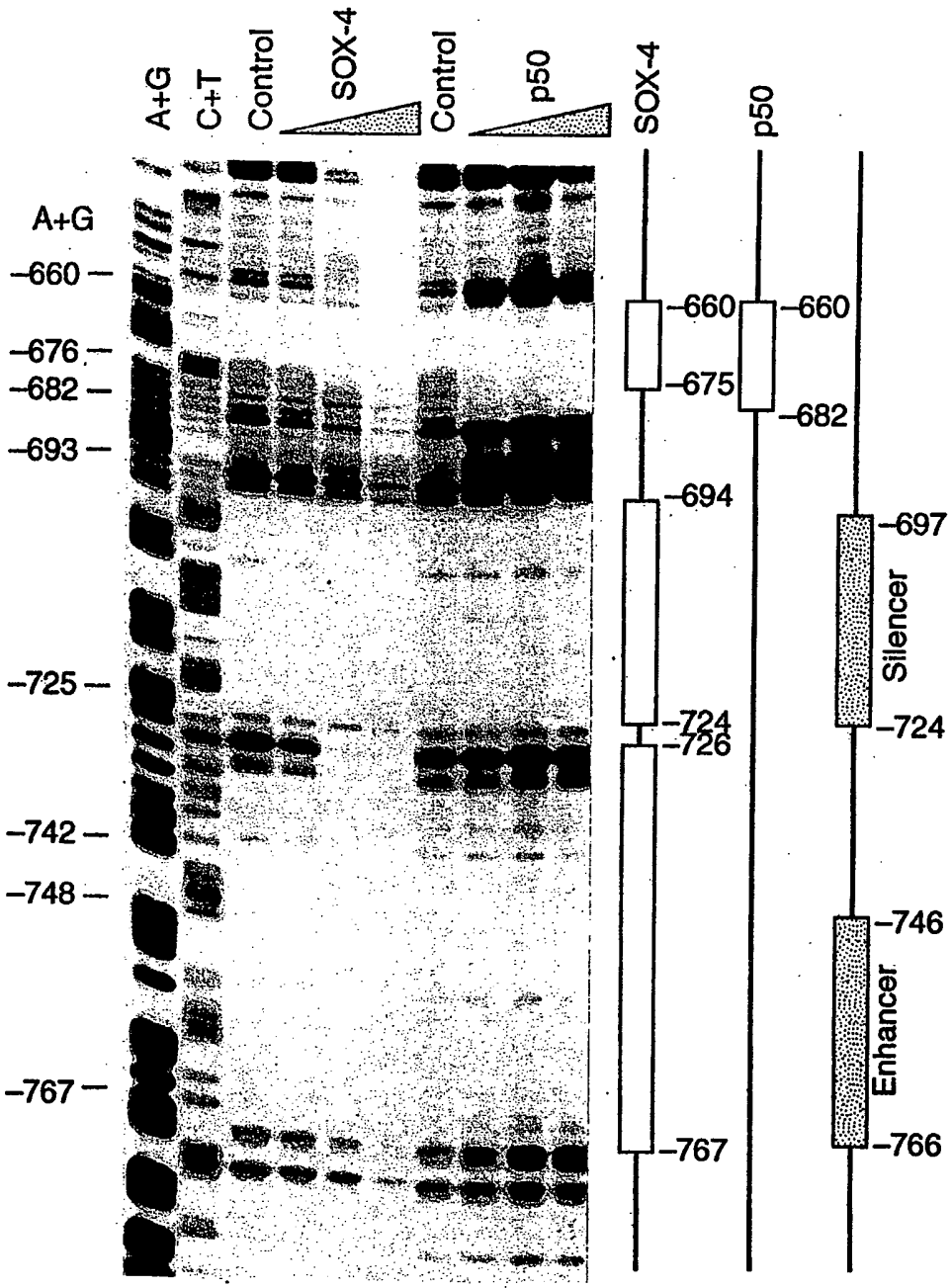

A+G
C+T
Control
SOX-4
Control
p50
SOX-4
p50
A+G
-660
-676
-682
-693
-725
-742
-748
-767
-660
-675
-694
-724
-726
-767
-660
-682
-697
Silencer
-724
-746
Enhancer
-766

FIG. 24B

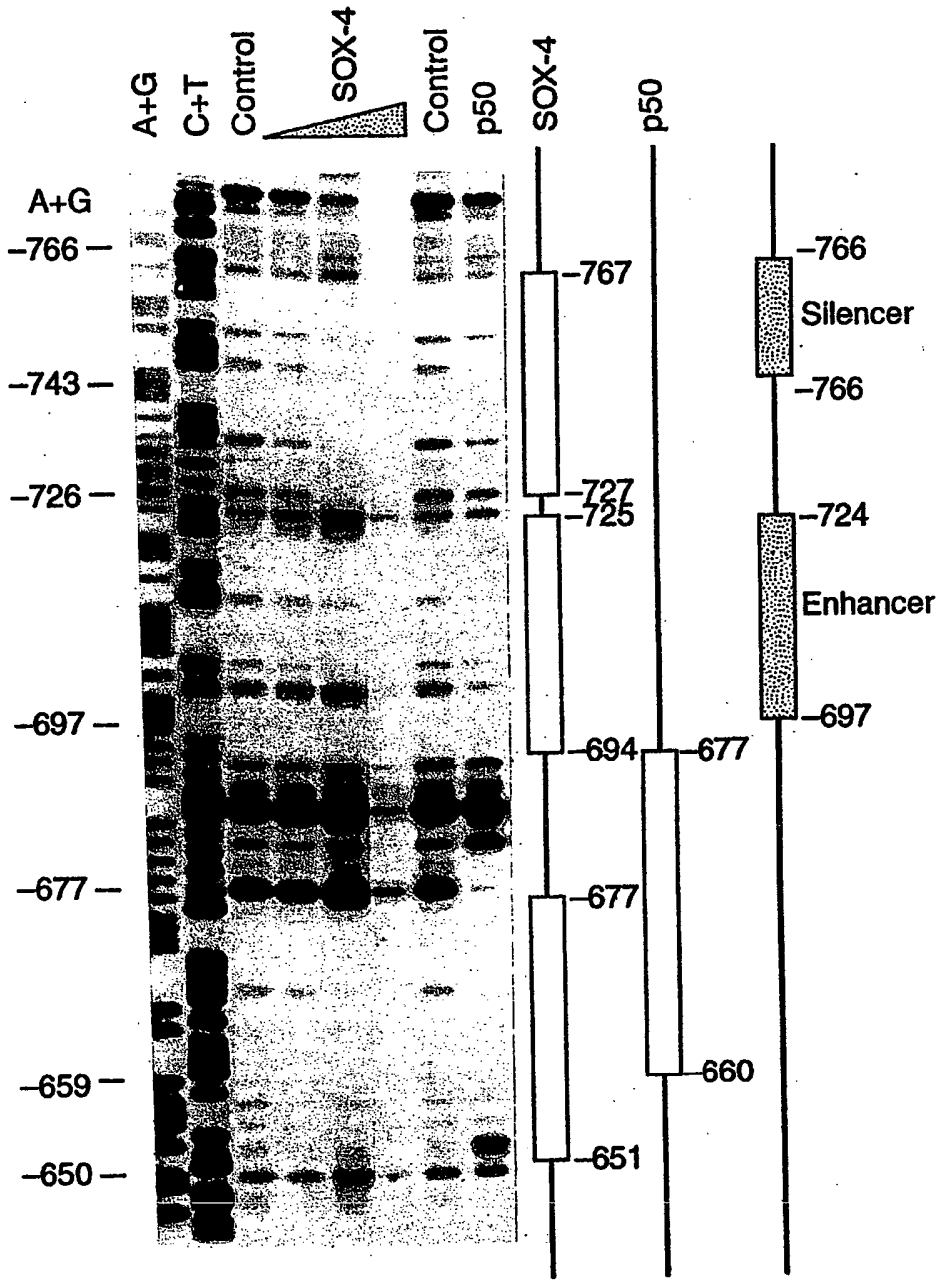

A+G
C+T
Control
SOX-4
Control
p50
SOX-4
p50
A+G
–766
–743
–726
–697
–677
–659
–650
–767
–727
–725
–694
–677
–651
–677
–660
–766
Silencer
–766
–724
Enhancer
–697

FIG. 25A

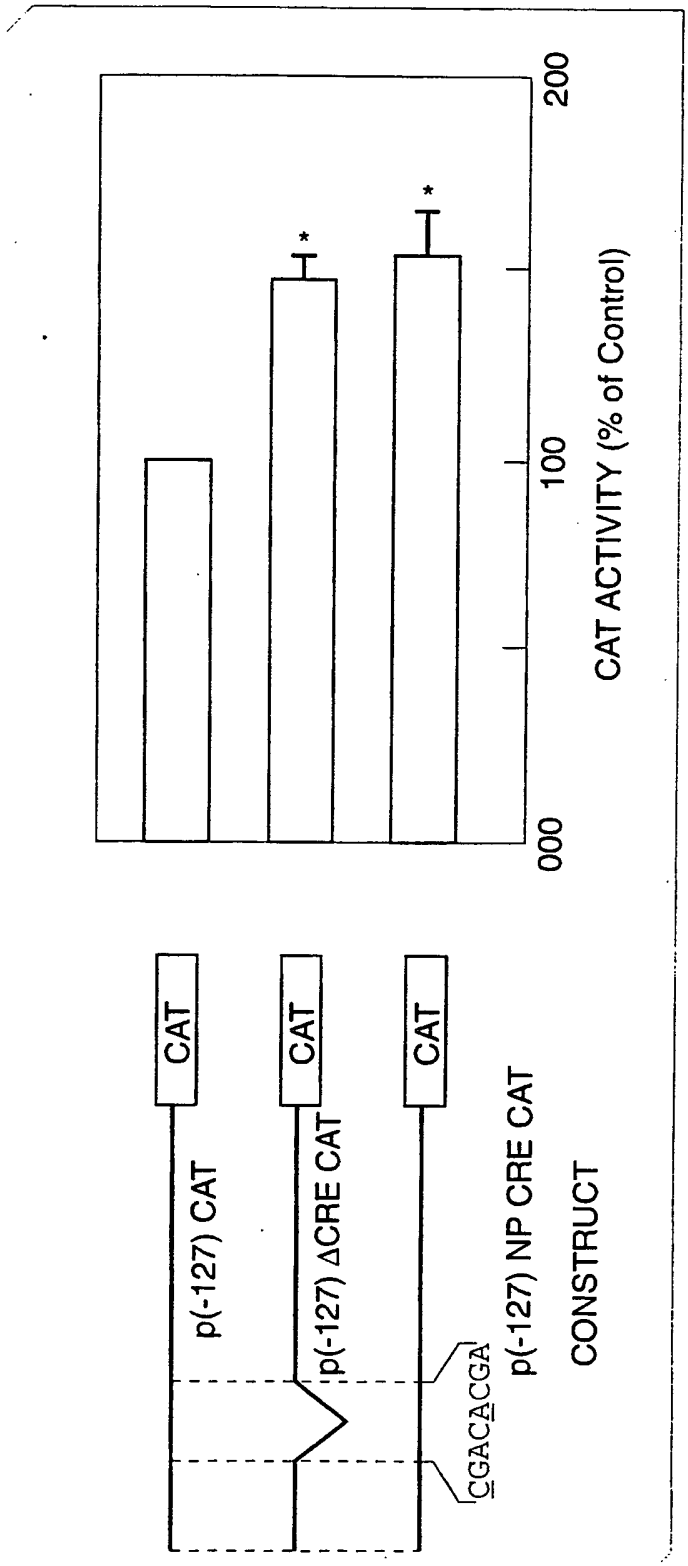

200
100
000
CAT ACTIVITY (% of Control)
*
*
CAT
CAT
CAT
p(-127) CAT
p(-127) ΔCRE CAT
p(-127) NP CRE CAT
CONSTRUCT
CGACACGA

FIG. 25B

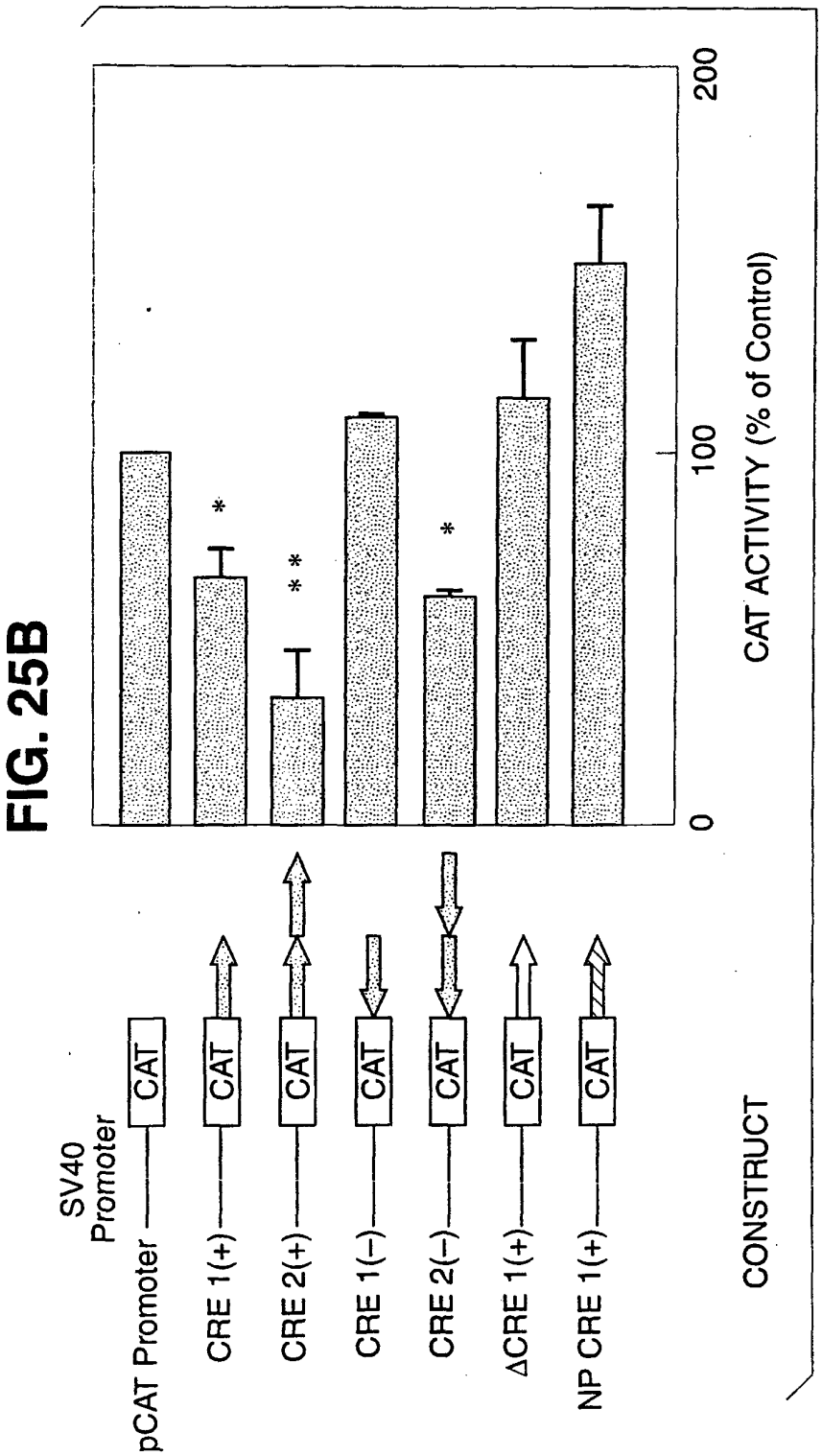

CAT ACTIVITY (% of Control)
0
100
200
CONSTRUCT
SV40 Promoter
pCAT Promoter
CAT
CRE 1(+)
CRE 2(+)
CRE 1(−)
CRE 2(−)
ΔCRE 1(+)
NP CRE 1(+)
*
**
*

FIG. 26A

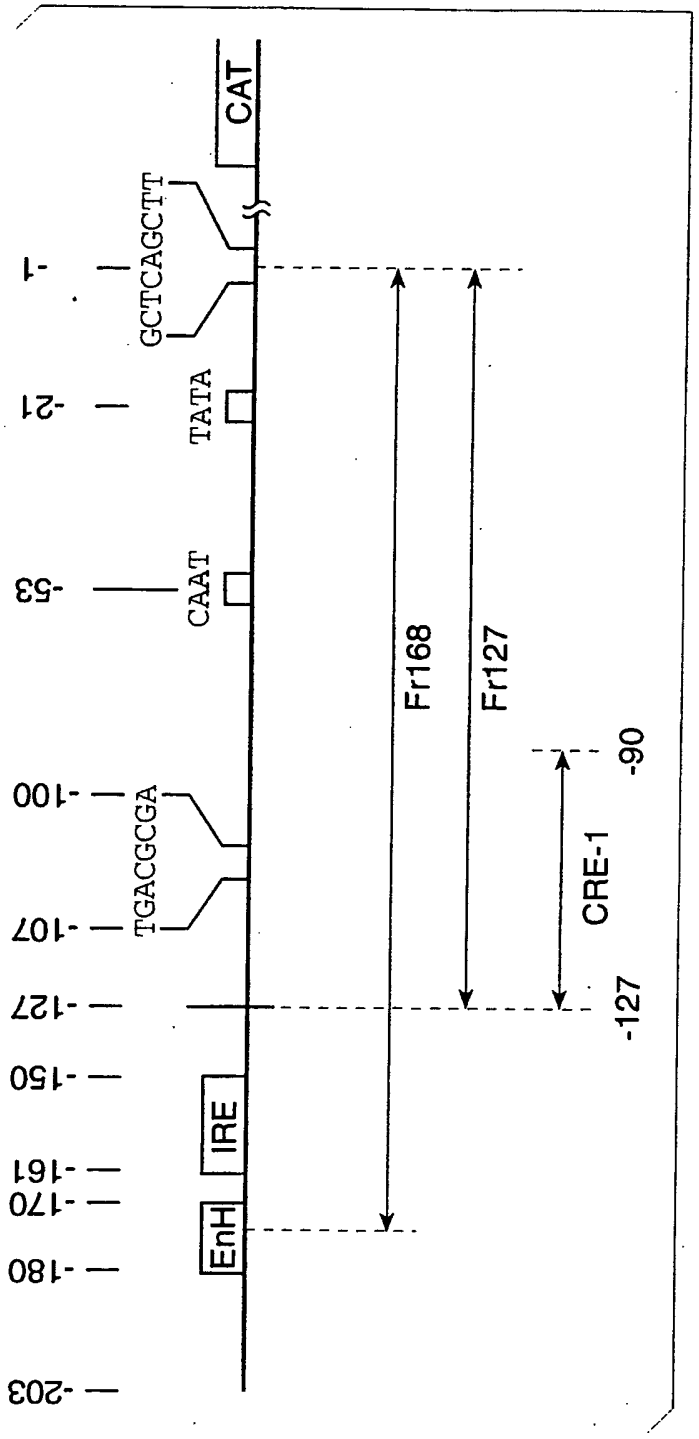

CAT
GCTCAGCTT
-1
-21
TATA
-53
CAAT
Fr168
Fr127
-90
CRE-1
-100
TGACGCGA
-107
-127
-150
IRE
-161
-170
EnH
-180
-203

FIG. 26B

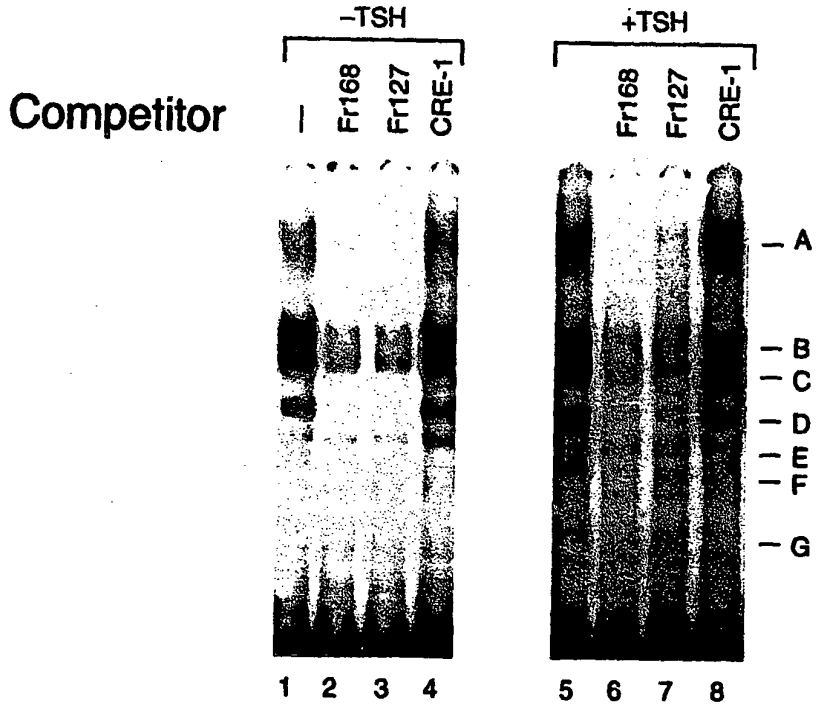

Competitor
-TSH
+TSH
—
Fr168
Fr127
CRE-1
Fr168
Fr127
CRE-1
A
B
C
D
E
F
G
1 2 3 4
5 6 7 8

FIG. 27A

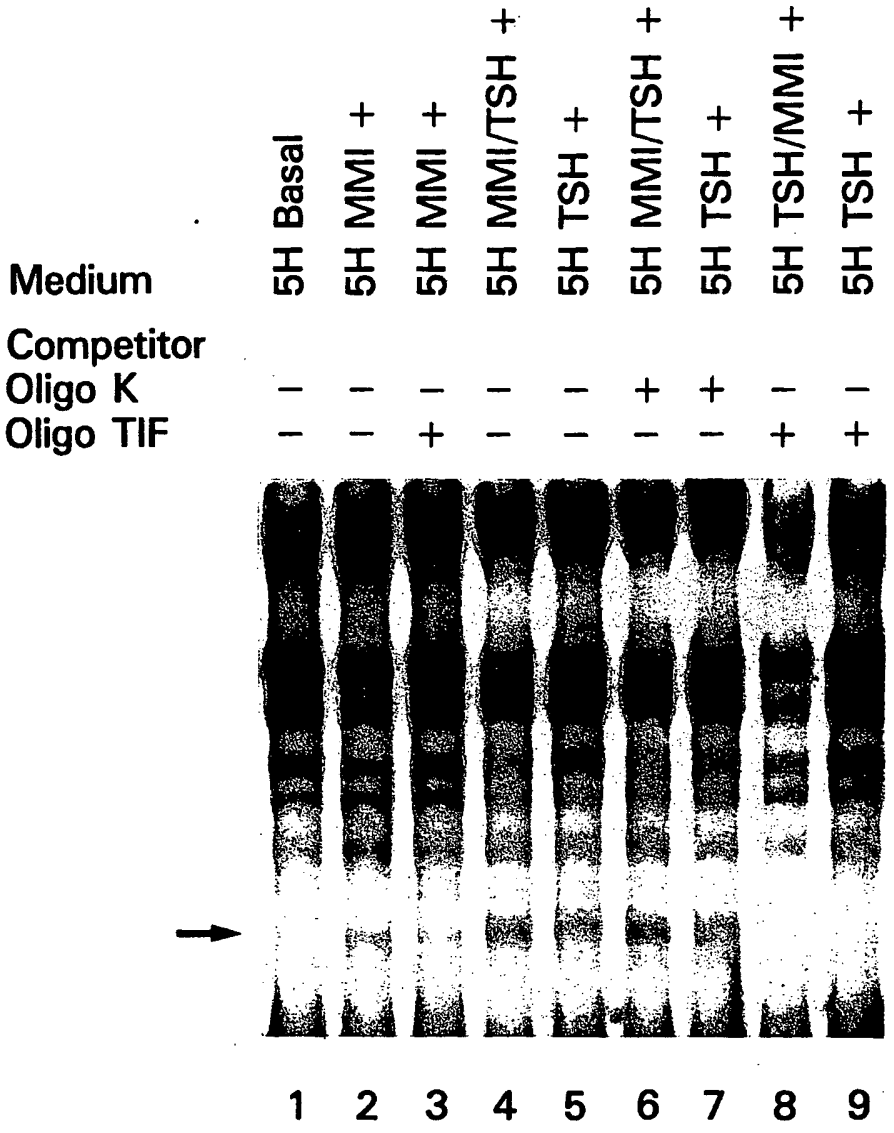

Medium
5H Basal
5H MMI +
5H MMI +
5H MMI/TSH +
5H TSH +
5H MMI/TSH +
5H TSH +
5H TSH/MMI +
5H TSH +
Competitor
Oligo K
Oligo TIF
1 2 3 4 5 6 7 8 9

FIG. 27B

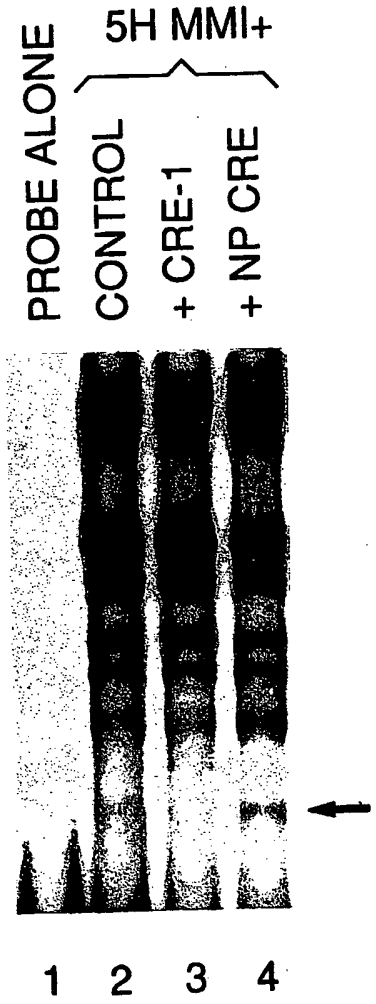


FIG. 28B

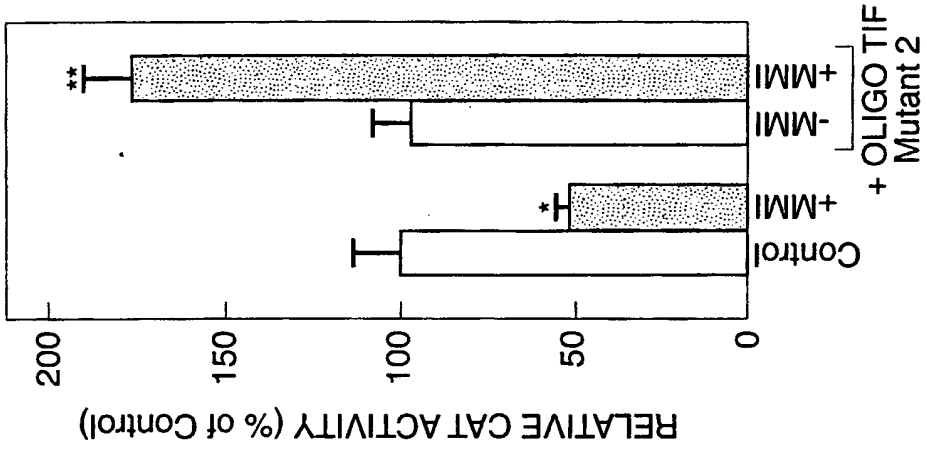
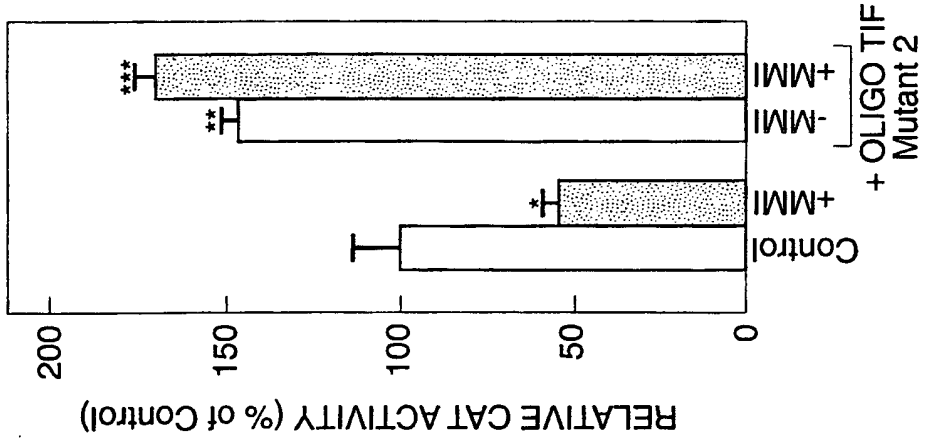


FIG. 28A



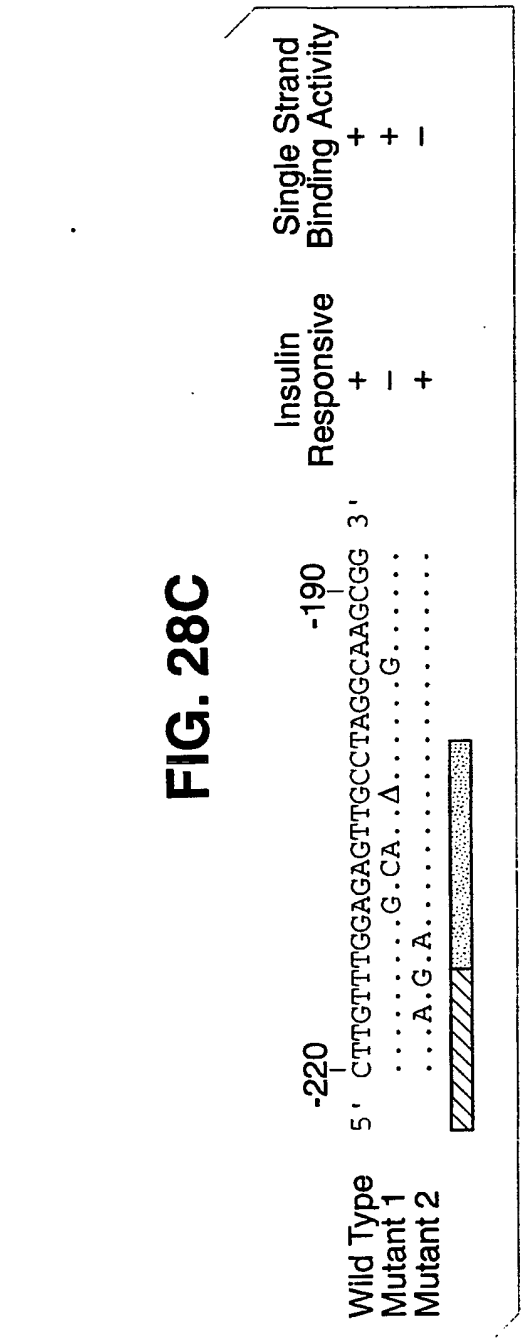

-220
-190
5' CTTGTTTGGAGAGTTGCCTAGGCAAGCGG 3'
Wild Type
Mutant 1
Mutant 2
Insulin Responsive
+
–
+
Single Strand Binding Activity
+
+
–

FIG. 28C

FIG. 29A

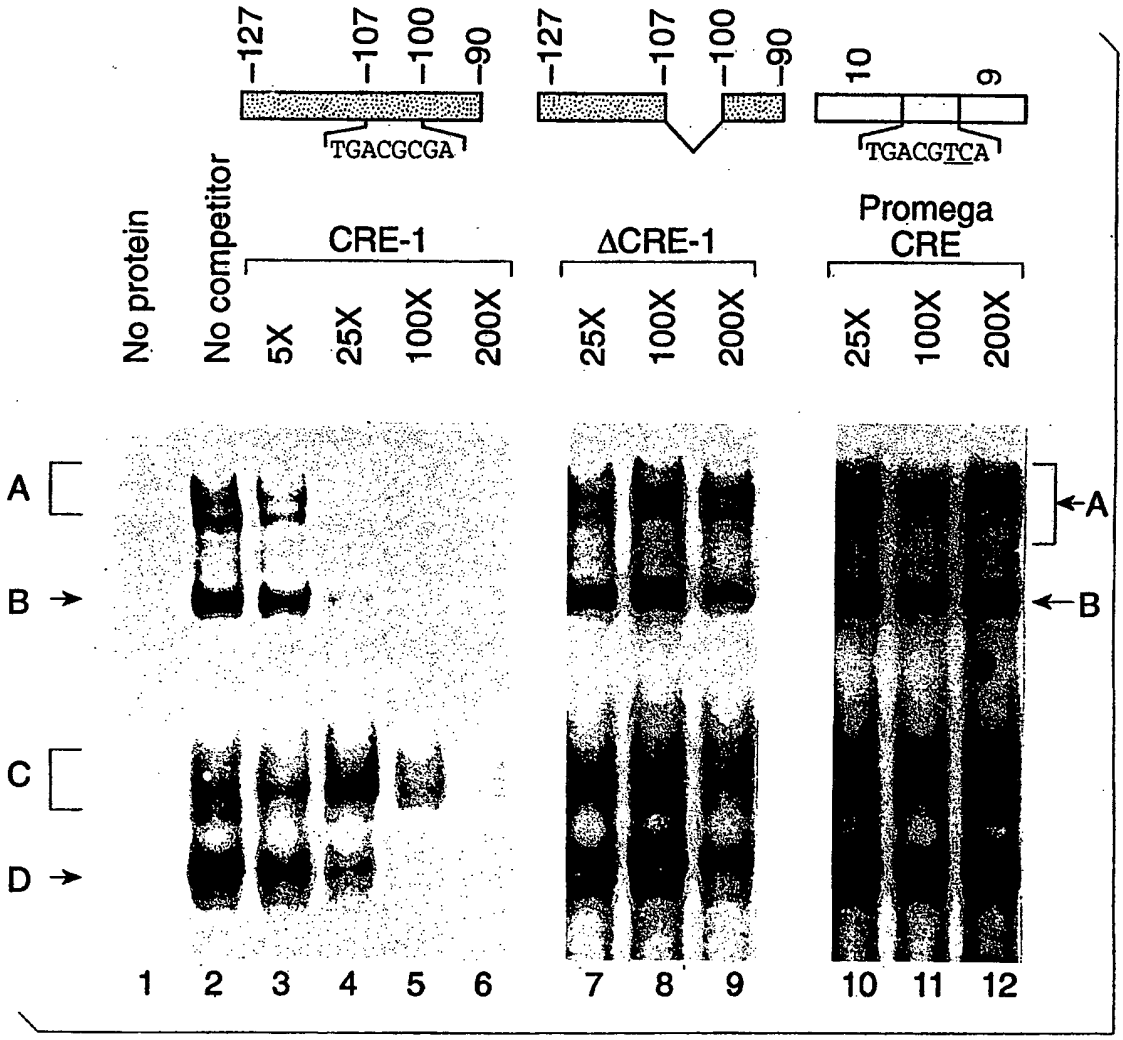

−127
−107
−100
−90
TGACGCGA
−127
−107
−100
−90
10
9
TGACGTCA
No protein
No competitor
CRE-1
5X
25X
100X
200X
ΔCRE-1
25X
100X
200X
Promega CRE
25X
100X
200X
A
B
C
D
A
B
1 2 3 4 5 6 7 8 9 10 11 12

FIG. 29B

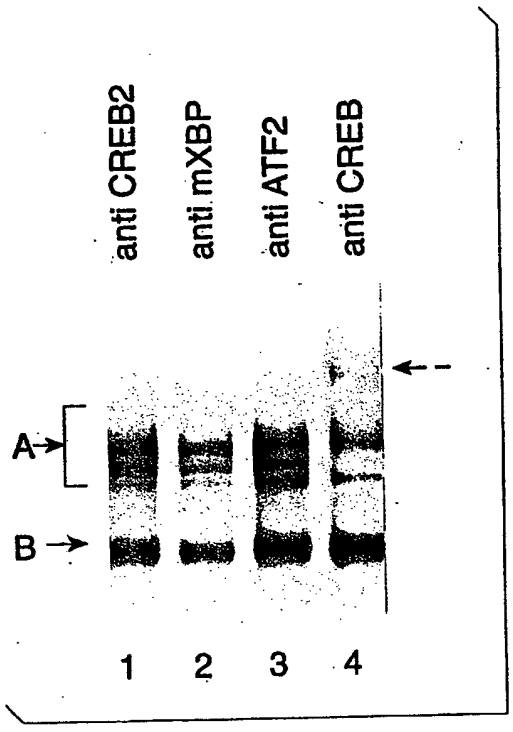


FIG. 30A

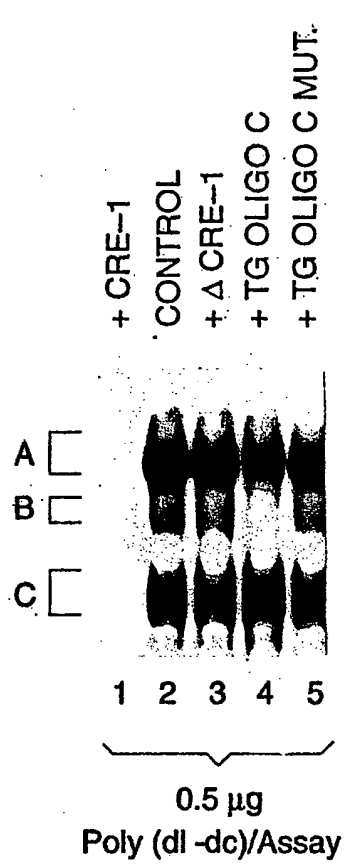


FIG. 30B

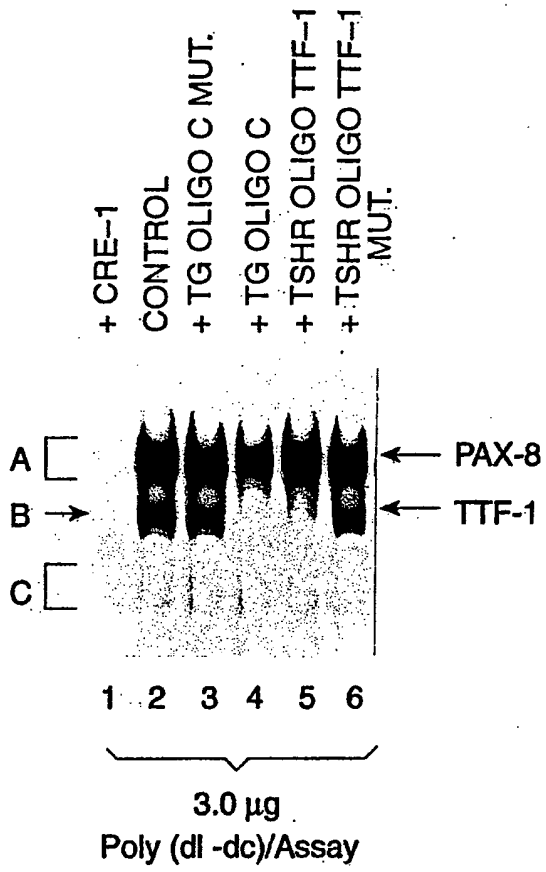


FIG. 30C

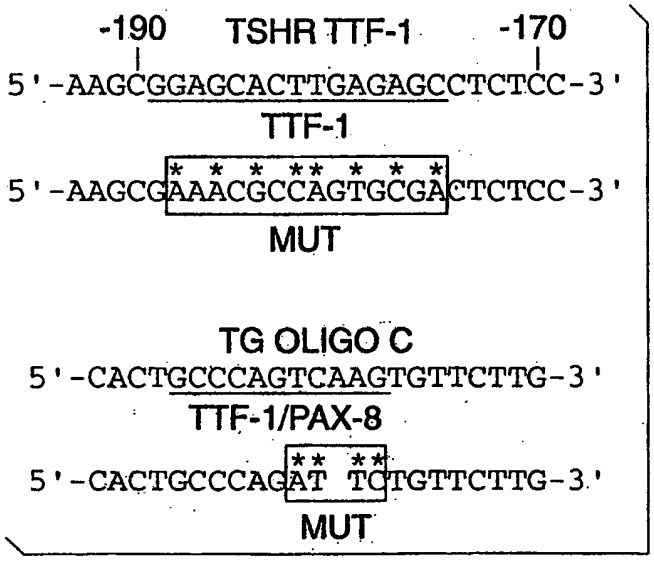


FIG. 31A

FIG. 31B

FIG. 31C

FIG. 32A

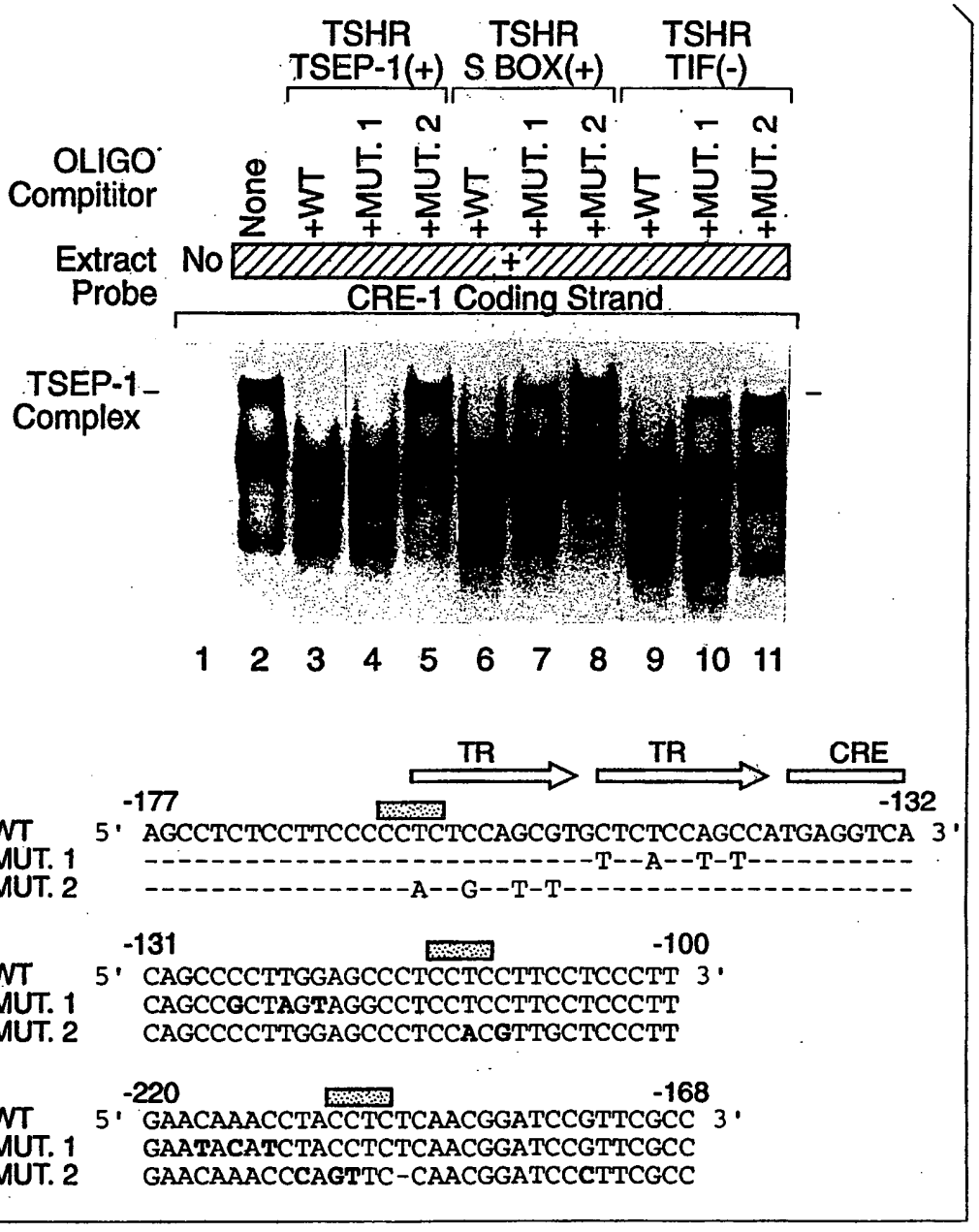

TSHR TSEP-1(+)
TSHR S BOX(+)
TSHR TIF(-)
OLIGO Compititor
None
+WT
+MUT. 1
+MUT. 2
+WT
+MUT. 1
+MUT. 2
+WT
+MUT. 1
+MUT. 2
Extract Probe
No
+
CRE-1 Coding Strand
TSEP-1 Complex
1 2 3 4 5 6 7 8 9 10 11
TR
TR
CRE
-177
-132
OLIGO TSEP-1 (+)
WT 5' AGCCTCTCCTTCCCCCTCTCCAGCGTGCTCTCCAGCCATGAGGTCA 3'
MUT. 1 ----------T--A--T-T----------
MUT. 2 ----------A--G--T-T----------
-131
-100
OLIGO S BOX (+)
WT 5' CAGCCCCTTGGAGCCCTCCTCCTTCCTCCCTT 3'
MUT. 1 CAGCCGCTAGTAGGCCTCCTCCTTCCTCCCTT
MUT. 2 CAGCCCCTTGGAGCCCTCCACGTTGCTCCCTT
-220
-168
OLIGO TIF (-)
WT 5' GAACAAACCTACCTCTCAACGGATCCGTTCGCC 3'
MUT. 1 GAATACATCTACCTCTCAACGGATCCGTTCGCC
MUT. 2 GAACAAACCCAGTTC-CAACGGATCCCTTCGCC

FIG. 32B

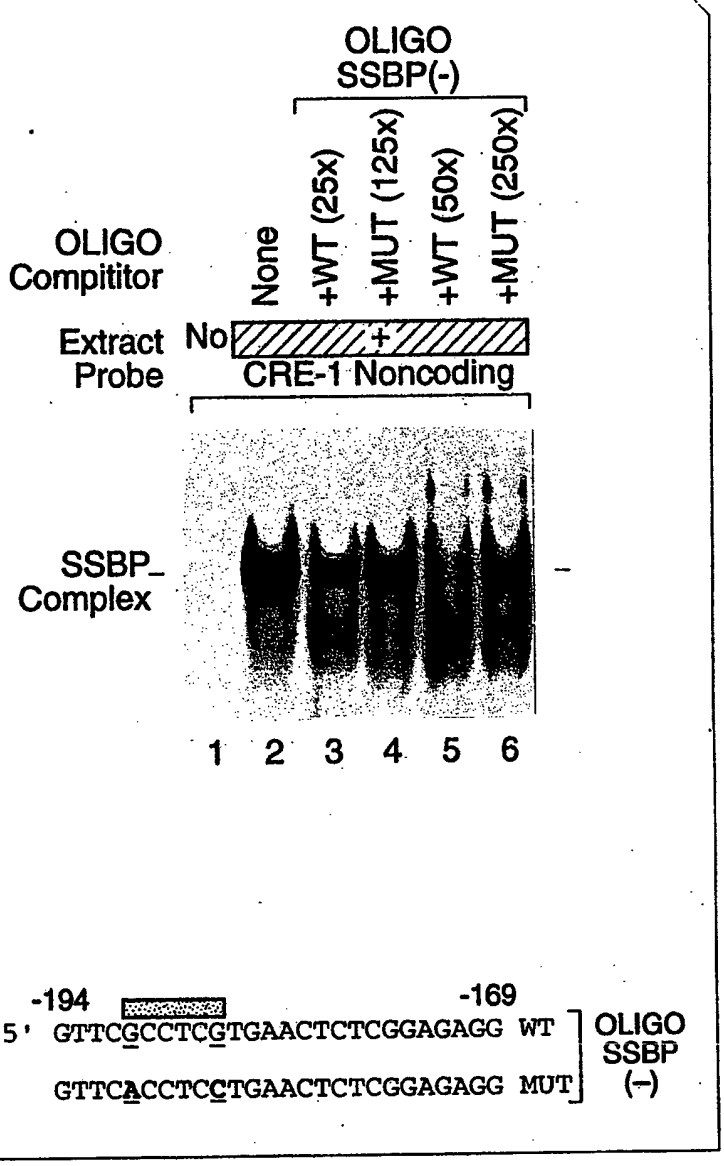
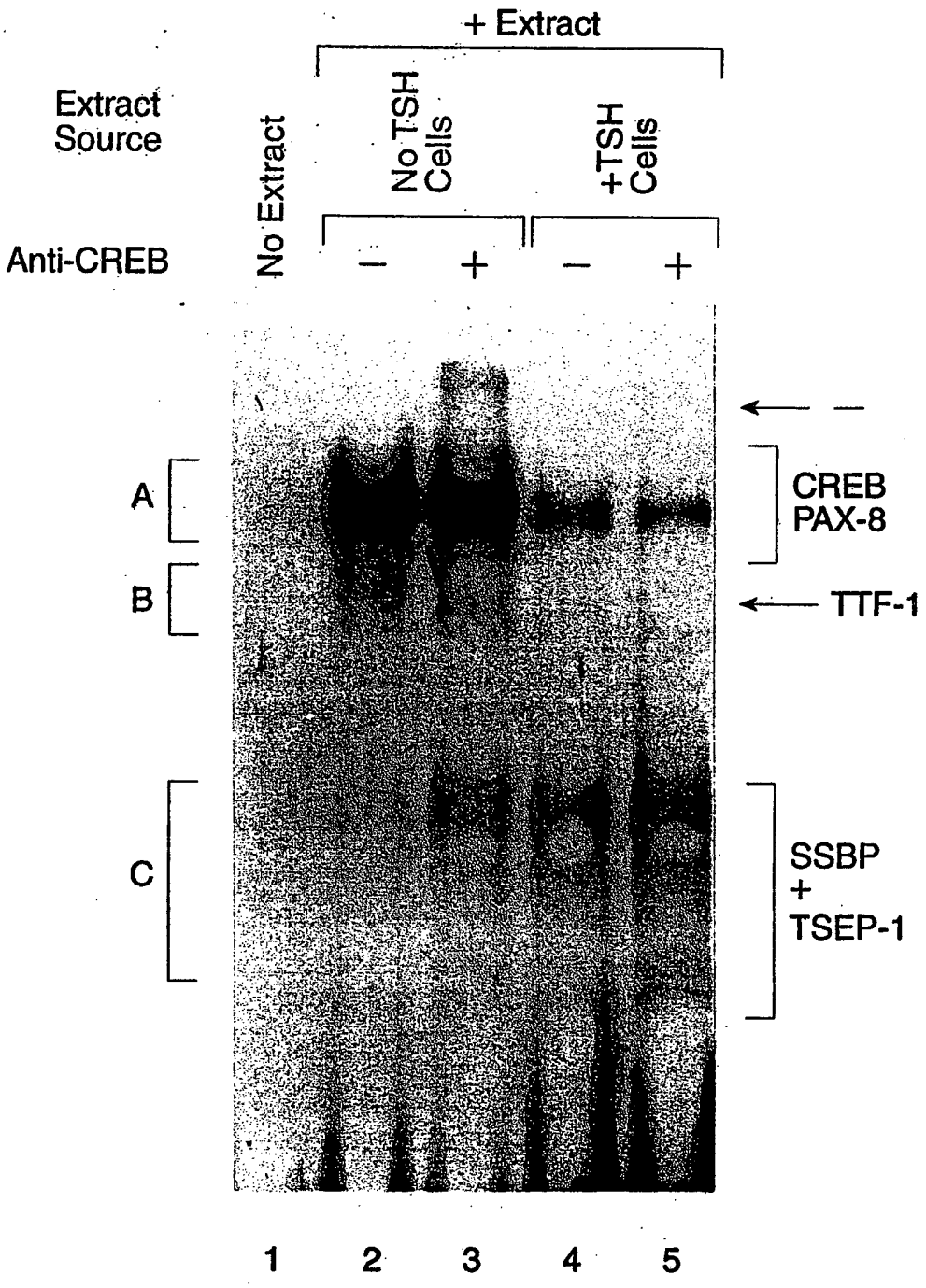
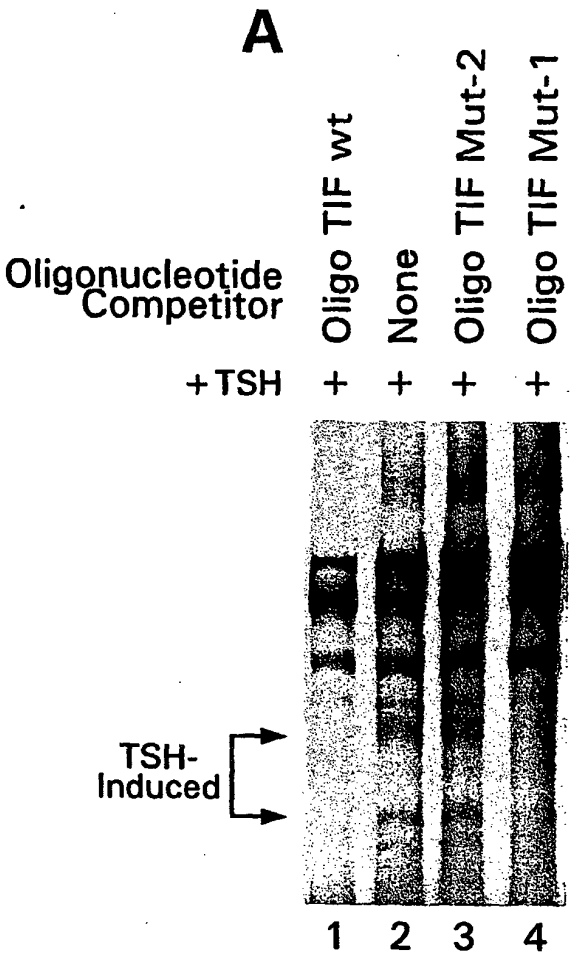

OLIGO
SSBP(-)
OLIGO
Compititor
None
+WT (25x)
+MUT (125x)
+WT (50x)
+MUT (250x)
Extract
Probe
No
+
CRE-1 Noncoding
SSBP_
Complex
1 2 3 4 5 6
TSHR
TSEP-1
BINDING
SITES
-194
-169
5' GTTCGCCTCGTGAACTCTCGGAGAGG WT
GTTCACCTCCTGAACTCTCGGAGAGG MUT
OLIGO
SSBP
(-)

FIG. 33



Probe: CRE-1 Double Strand
Poly(dI-dC) 3µg/Assay

FIG. 34A



Probe: Fr 168

FIG. 34B

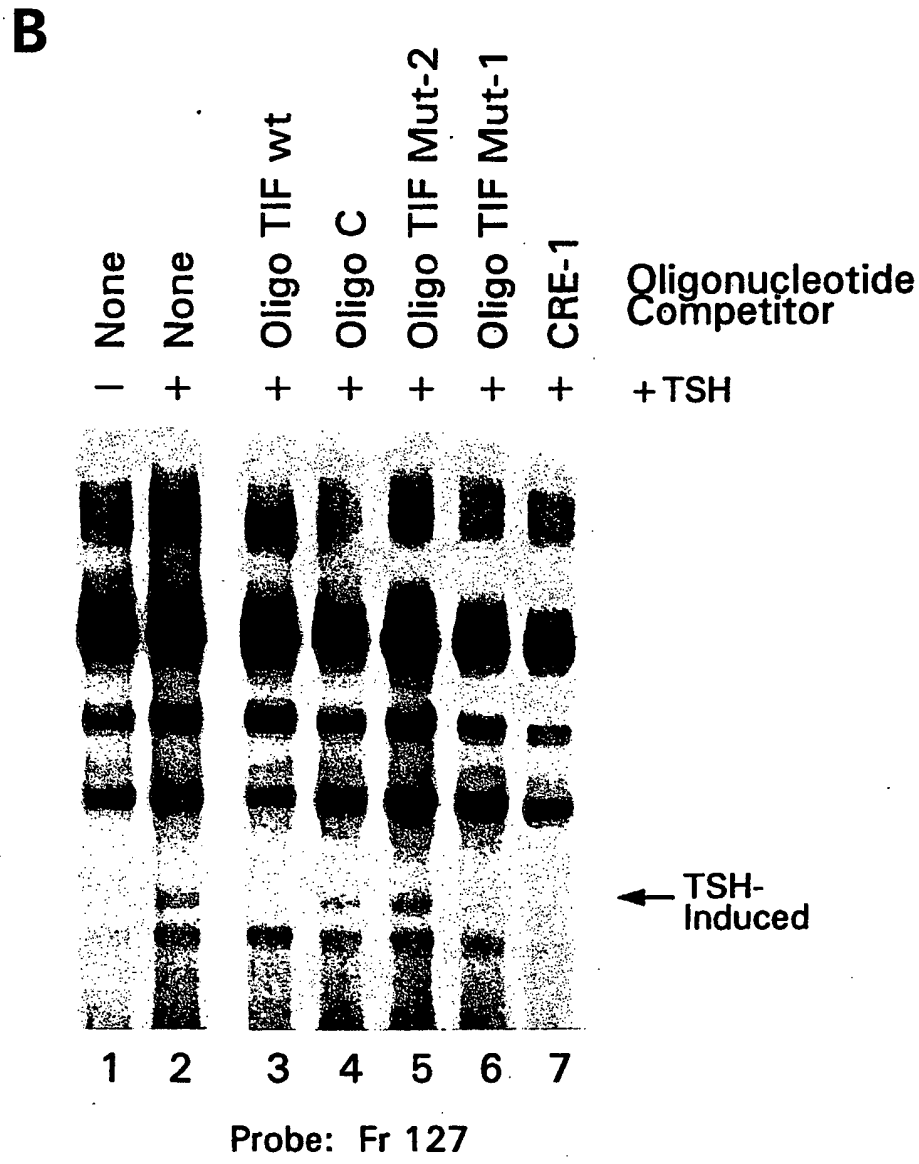

B
None
None
Oligo TIF wt
Oligo C
Oligo TIF Mut-2
Oligo TIF Mut-1
CRE-1
Oligonucleotide Competitor
–
+
+
+
+
+
+
+TSH
TSH-Induced
1
2
3
4
5
6
7
Probe: Fr 127

FIG. 35A

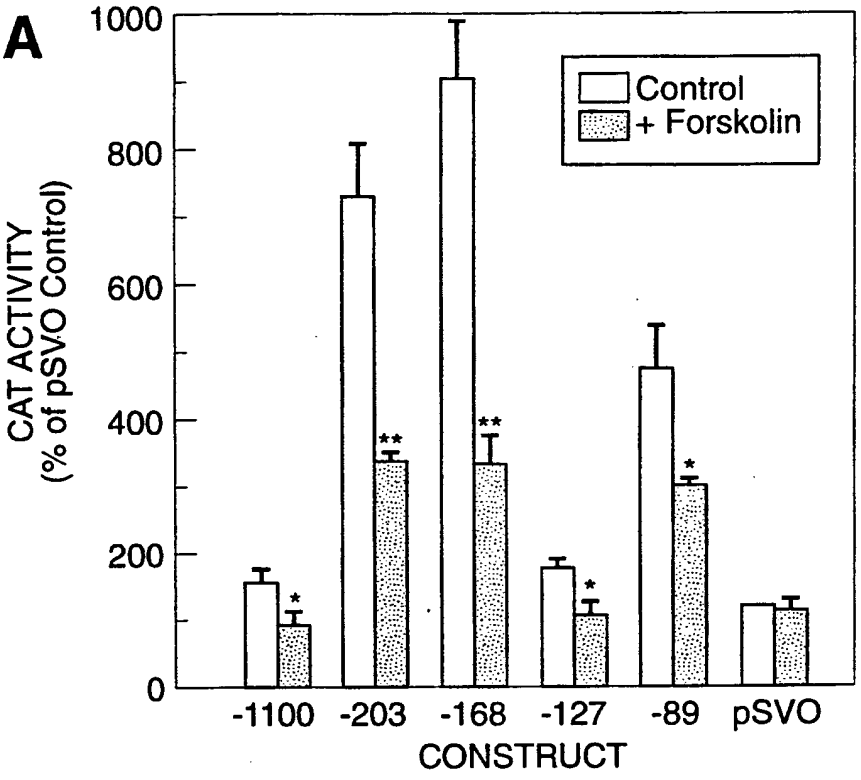


FIG. 35B

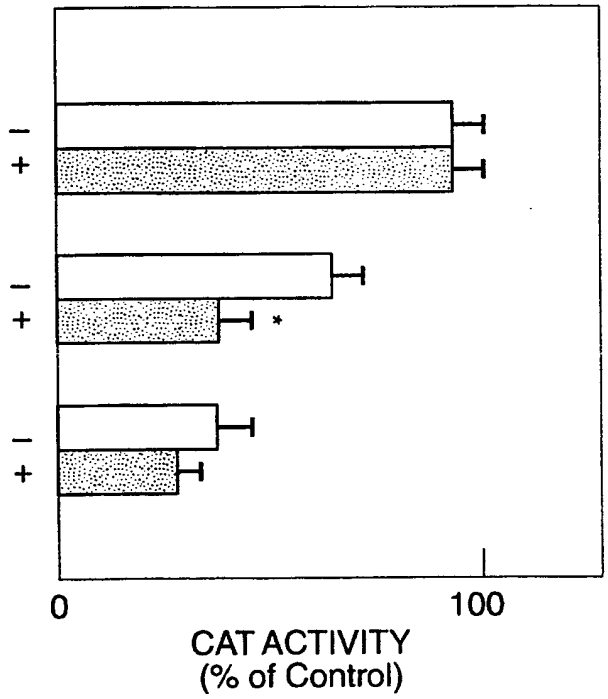


FIG. 35C

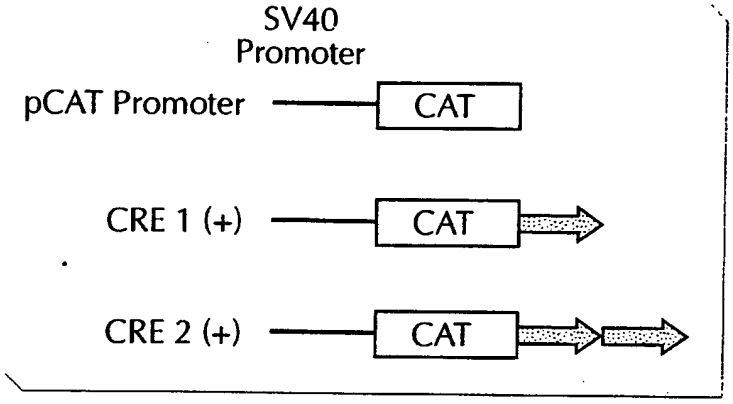


FIG. 37C

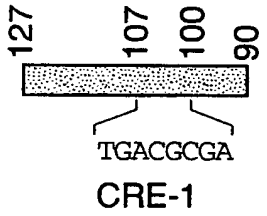


FIG. 37D

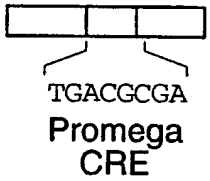


FIG. 38A

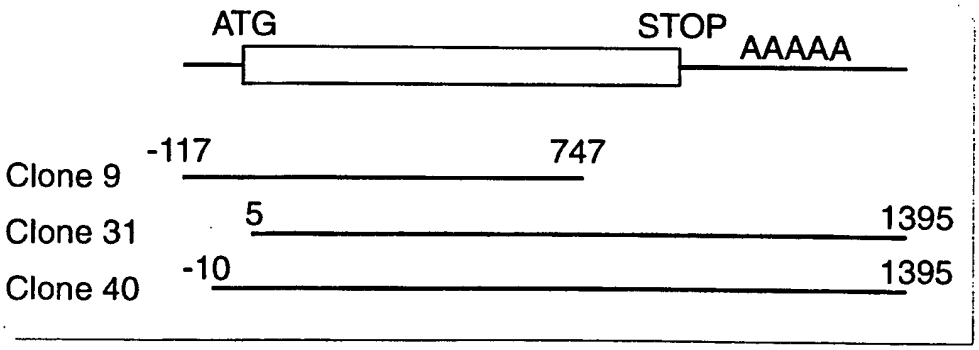
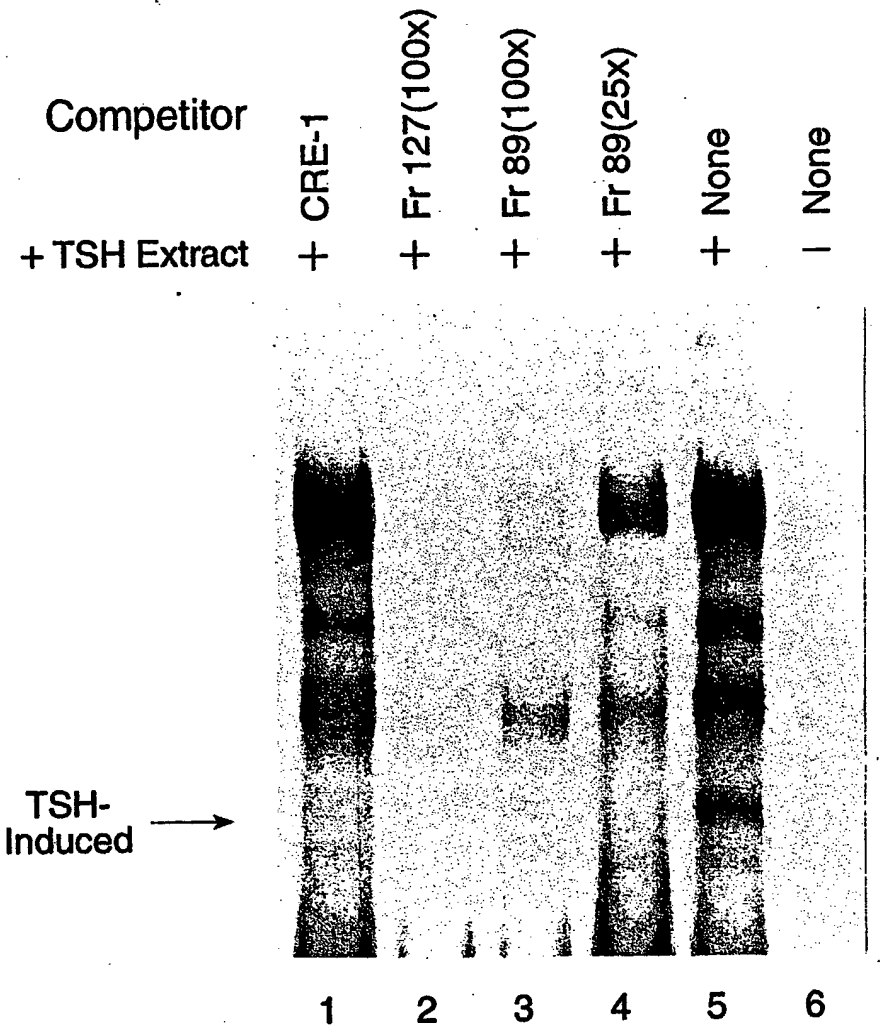


FIG. 36


Competitor
+ TSH Extract
+ CRE-1
+ Fr 127(100x)
+ Fr 89(100x)
+ Fr 89(25x)
+ None
– None
TSH-Induced
1
2
3
4
5
6

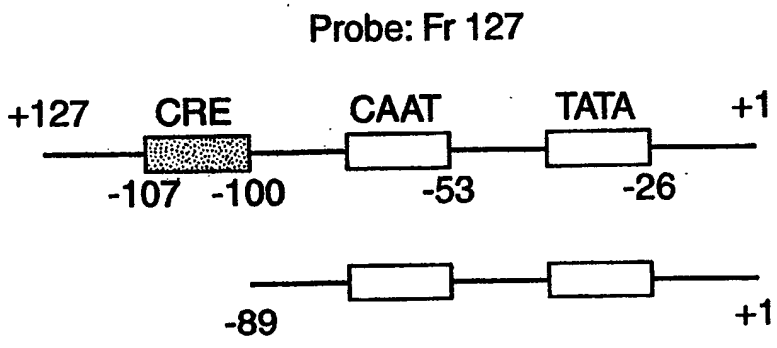

Probe: Fr 127
+127
CRE
CAAT
TATA
+1
-107
-100
-53
-26
-89
+1

FIG. 37A

FIG. 37B

B.

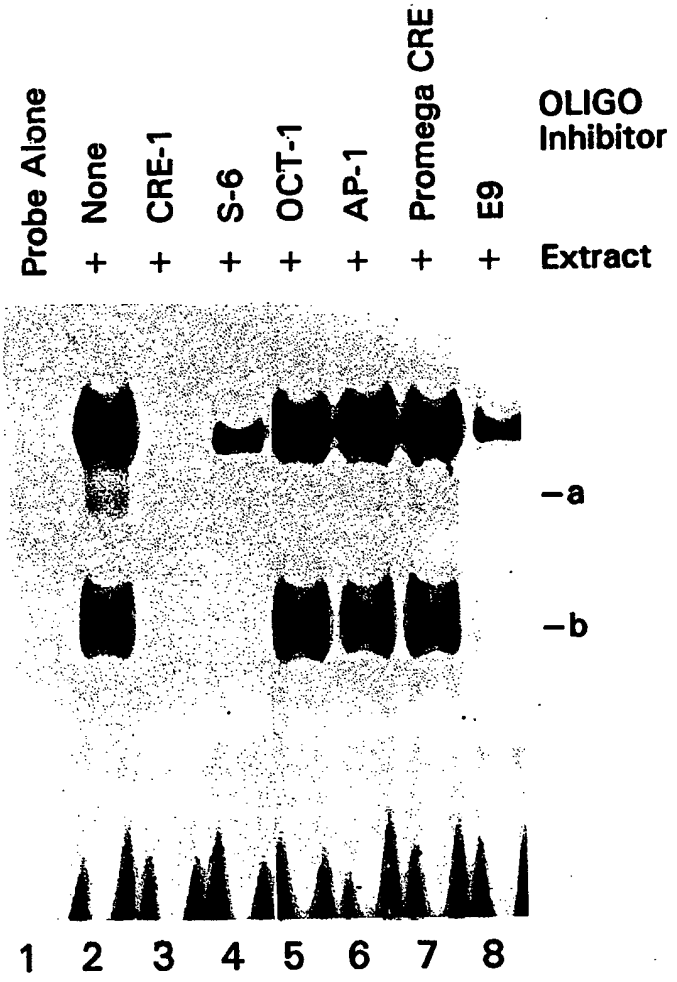

Probe Alone
None
CRE-1
S-6
OCT-1
AP-1
Promega CRE
E9
OLIGO Inhibitor
+ + + + + + + Extract
–a
–b
1 2 3 4 5 6 7 8
38bp Downstream Silencer (CRE-1)

FIG. 38B

```
GAATTCCGGT CTCACTGGTC TACCTTGCTC TCCTGCACCC TGGTTGTCAG  -68

CACCCACCAT CACACCCGGG AGGAGCCGCA GCCGTCGCCG CCGGCCCCAG  -18

TCACCATCAC CGCAACCATG AGCAGCGAGG CCGAGACCCA GCAGCCGCCC   33
                  Met SerSerGluA laGluThrGl nGlnPrcPro

GCCGCCCCCG CCGCCGCCCT CAGCGCCGCC GACACCAAGC CCGGCTCCAC   83
AlaAlaProA laAlaAlaLe uSerAlaAla AspThrLysP roGlySerTh

GGGCAGCGGC GCGGGTAGTG GCGGCCCGGG CGGCCTCACA TCGGCGGCGC  133
rGlySerGly AlaGlySerG lyGlyProGl yGlyLeuThr SerAlaAlaP

CCGCCGGCGG GGACAAGAAG GTCATCGCAA CGAAGGTTTT GGGAACAGTA  183
roAlaGlyGl yAspLysLys ValIleAlaT hrLysValLe uGlyThrVal

AAATGGTTCA ATGTAAGGAA CGGATACGGT TTCATCAACA GGAATGACAC  233
LysTrpPheA snValArgAs nGlyTyrGly PheIleAsnA rgAsnAspTh

CAAGGAAGAC GTATTTGTAC ACCAGACGGC CATAAAGAAG AATAACCCCA  283
rLysGluAsp ValPheValH isGlnThrAl aIleLysLys AsnAsnProA

GGAAGTACCT TCGCAGTGTA GGAGATGGAG AGACTGTGGA GTTTGATGTT  333
rgLysTyrLe uArgSerVal GlyAspGlyG luThrValGl uPheAspVal

GTTGAAGGAG AAAAGGGTGC GGAGGCAGCT AATGTTACAG GCCCTGGTGG  383
ValGluGlyG luLysGlyAl aGluAlaAla AsnValThrG lyProGlyGl

AGTTCCAGTT CAAGGCAGTA AATACGCAGC AGACCGTAAC CATTATAGGC  433
yValProVal GlnGlySerL ysTyrAlaAl aAspArgAsn HisTyrArgA

GCTATCCACG TCGTAGGGGT CCTCCACGCA ATTACCAGCA AAATTACCAG  483
rgTyrProAr gArgArgGly ProProArgA snTyrGlnGl nAsnTyrGln

AATAGTGAGA GTGGGGAAAA GAATGAAGGA TCGGAAAGCG CTCCTGAAGG  533
AsnSerGluS erGlyGluLy sAsnGluGly SerGluSerA laProGluGl

CCAGGCCCAA CAACGCCGGC CCTATCGCAG CCGAAGGTTC CCACCTTACT  583
yGlnAlaGln GlnArgArgP roTyrArgAr gArgArgPhe ProProTyrT

ACATGCGGAG GCCCTATGCG CGTCGACCAC AGTATTCCAA CCCCCCTGTG  633
yrMetArgAr gProTyrAla ArgArgProG lnTyrSerAs nProProVal
```

56/64

FIG. 38B'

```
CAAGGAGAAG TGATGGAGGG TGCTGACAAC CAGGGTGCAG GAGAGCAAGG  683
GlnGlyGluV alMetGluGl yAlaAspAsn GlnGlyAlaG lyGluGlnGl

TAGACCAGTG AGACAGAATA TGTATCGGGG TTACAGACCA CGATTCCGCA  733
yArgProVal ArgGlnAsnM etTyrArgGl yTyrArgPro ArgPheArgA

GGGGCCCTCC TCGCCCAAGA CAGCCTAGAG AGGATGGCAA TGAAGAGGAC  783
rgGlyProPr oArgGlnArg GlnProArgG luAspGlyAs nGluGluAsp

AAAGAAAATC AAGGAGATGA GACCCAAGGT CAGCAGCCAC CTCAACGTCG  833
LysGluAsnG lnGlyAspGl uThrGlnGly GlnGlnProP roGlnArgAr

GTATCGCCGC AACTTCAATT ACCGACGCAG ACGCCCAGAG AACCCTAAAC  883
gTyrArgArg AsnPheAsnT yrArgArgAr gArgProGlu AsnProLysP

CACAAGATGG CAAAGAGACA AAAGCAGCCG ATCCACCAGC TGAGAATTCG  933
roGlnAspGl yLysGluThr LysAlaAlaA spProProAl aGluAsnSer

TCCGCTCCCG AGGCTGAGCA GGGCGGGGCT GAGTAAATGC CGGCTTACCA  983
SerAlaProG luAlaGluGl nGlyGlyAla Glu

TCTCTACCAT CATCCGGTTT GGTCATCCAA CAAGAAGAAA TGAATATGAA 1033

ATTCCAGCAA TAAGAAATGA ACAAAGATTG GAGCTGAAGA CCTTAAGTGC 1083

TTGCTTTTTG CCCGTTGACC AGATCCACTA GAACTGTCTG CATTATCTAT 1133

GCAGCATGGG GTTTTTATTA TTTTTACCTA AAGATGTCTC TTTTTGGTAA 1183

TGACAAACGT GTTTTTTAAG AAAAAAAAAA AGGCCTGGTT TTTCTCAATA 1233

CACCTTTAAC GGTTTTTAAA TTGTTTCATA TCTGGTCAAG TTGAGATTTT 1283

TAAGAACTTC ATTTTTAATT TGTAATAAAG TTTACAACTT GATTTTTTCA 1333

AAAAAGTCAA CAAACTGCAA GCACCTGTTA ATAAAGGTCT TAAATAATAA 1383

AAAACGGAAT TC                                          1395
```

FIG. 39A

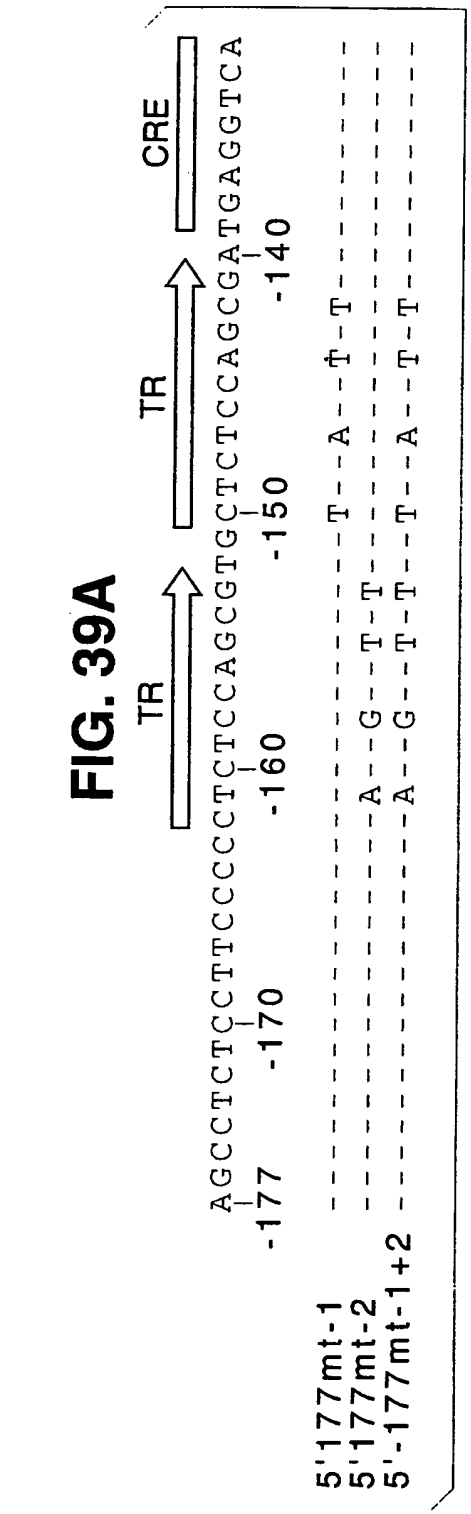


FIG. 39C

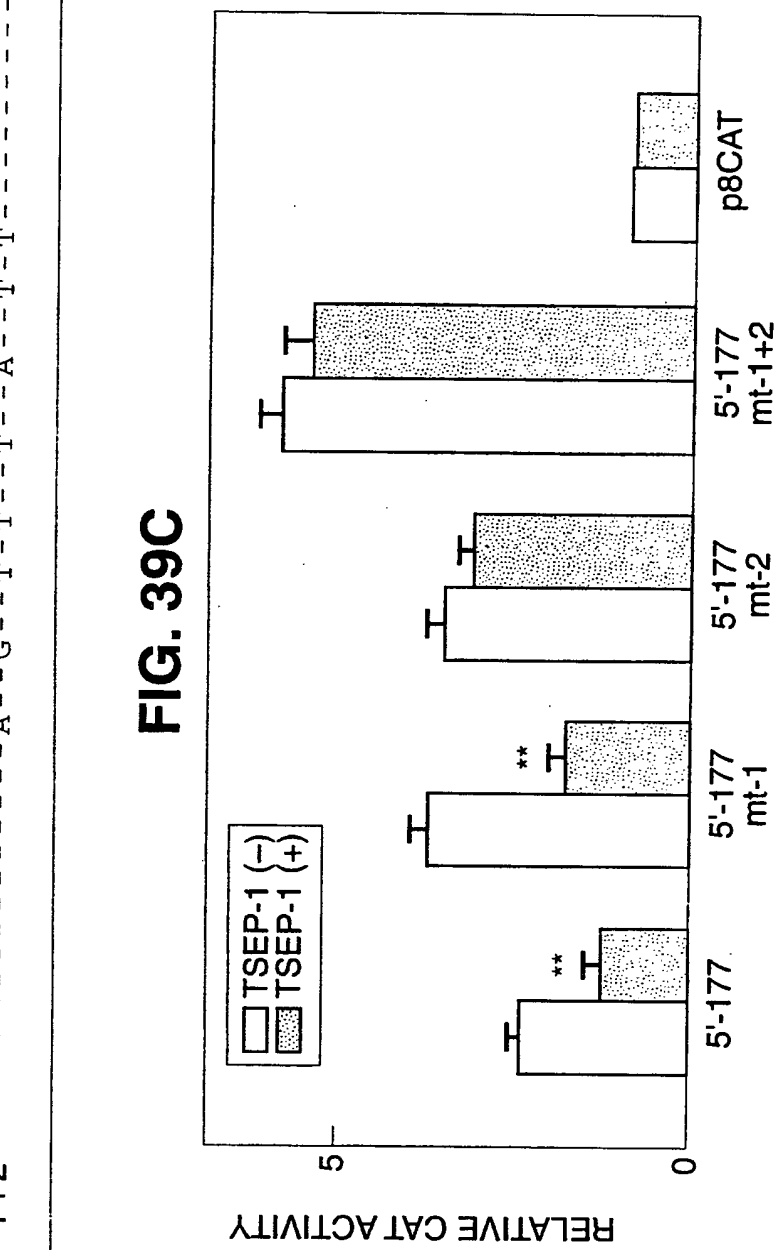


FIG. 39B

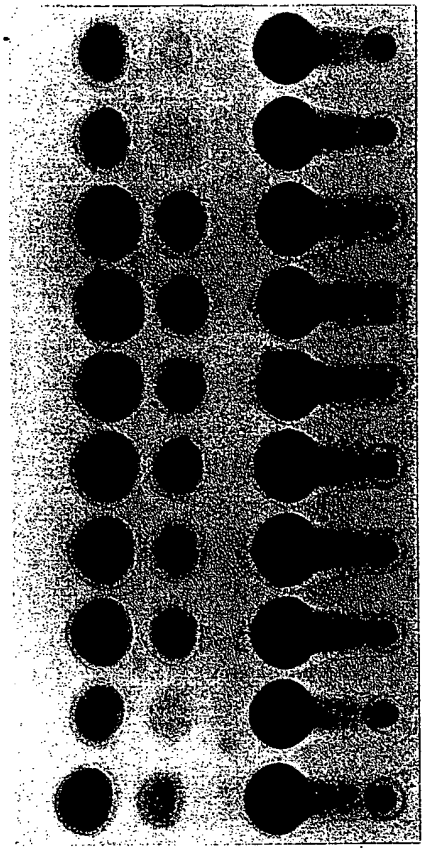


FIG. 40A

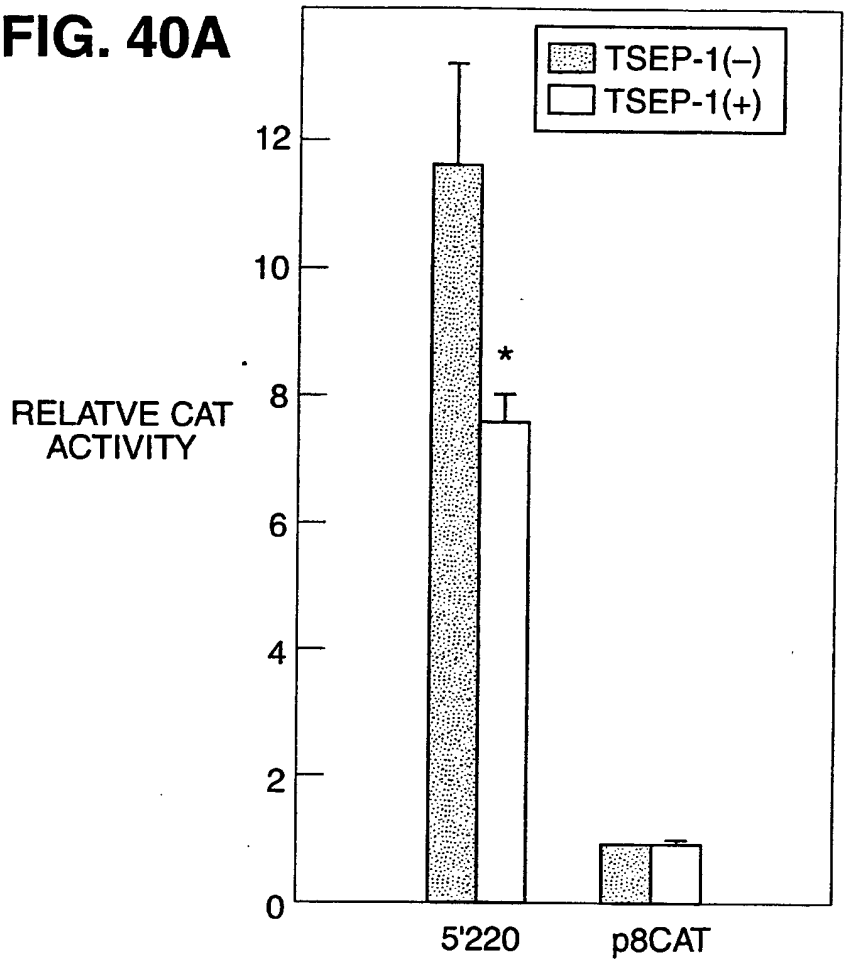


FIG. 40A'

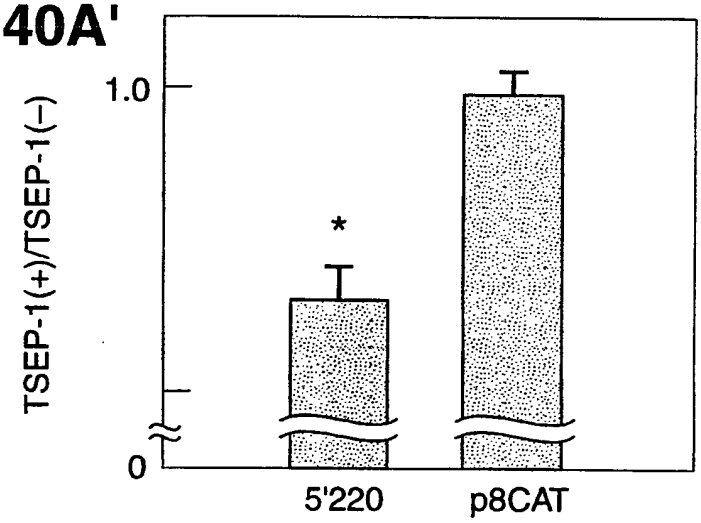


FIG. 40B

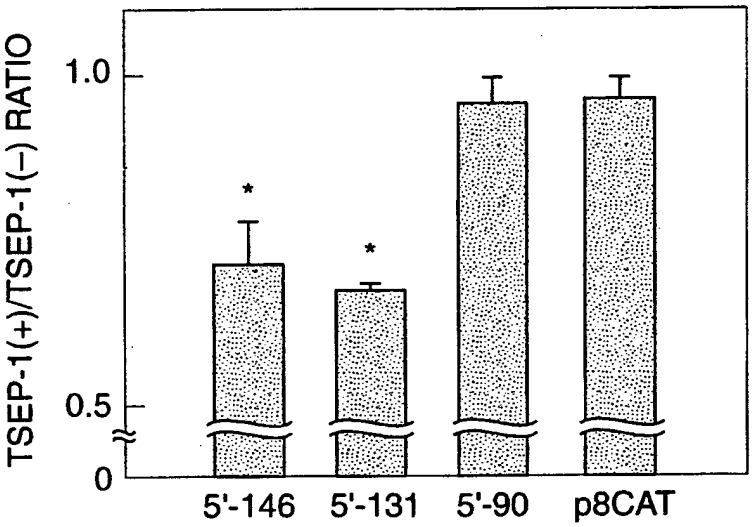


FIG. 40C

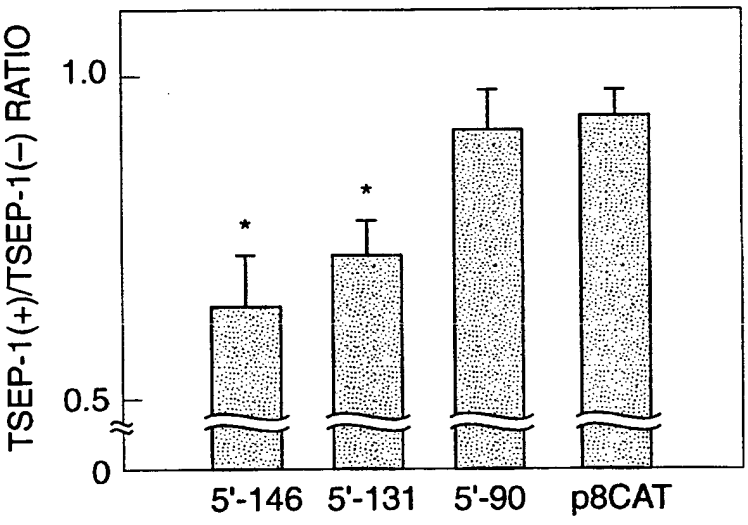


FIG. 40D

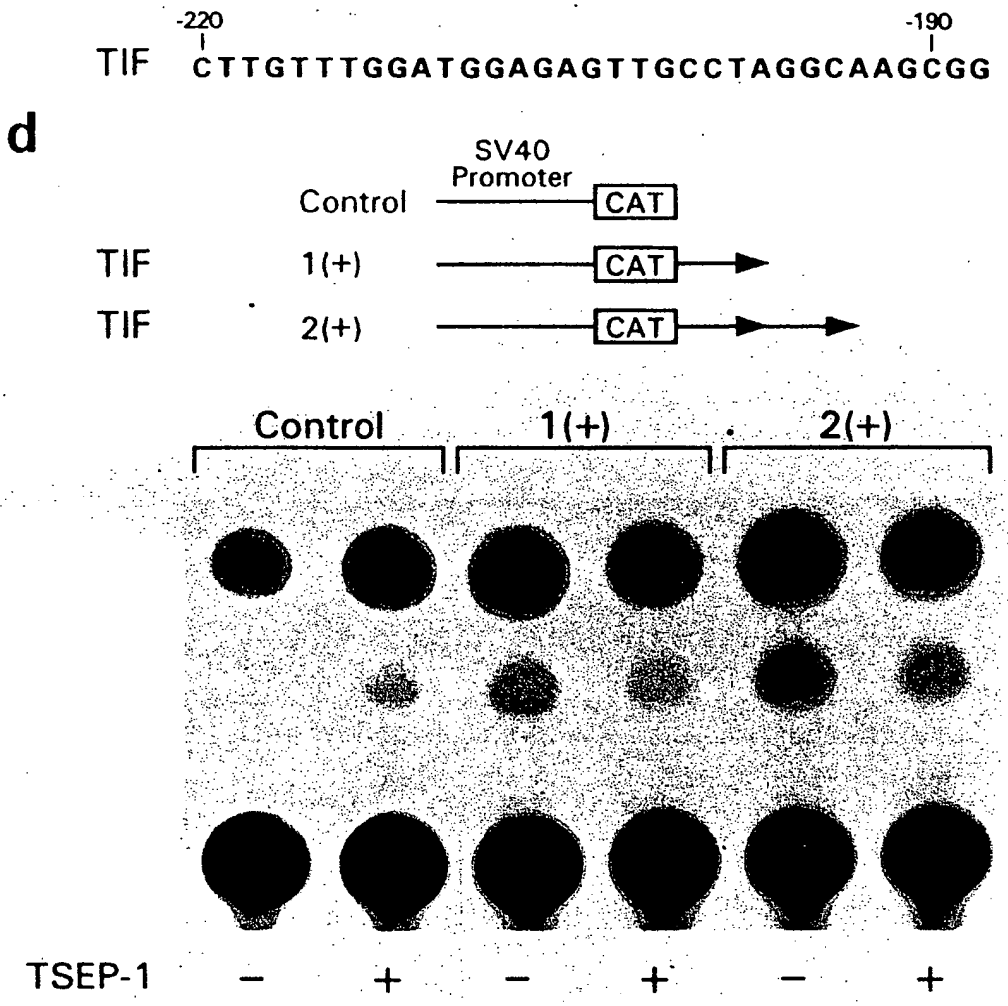


FIG. 40D'

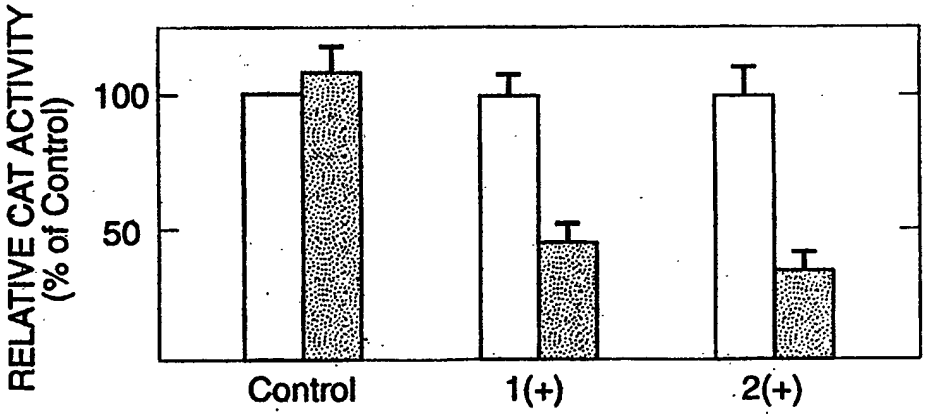
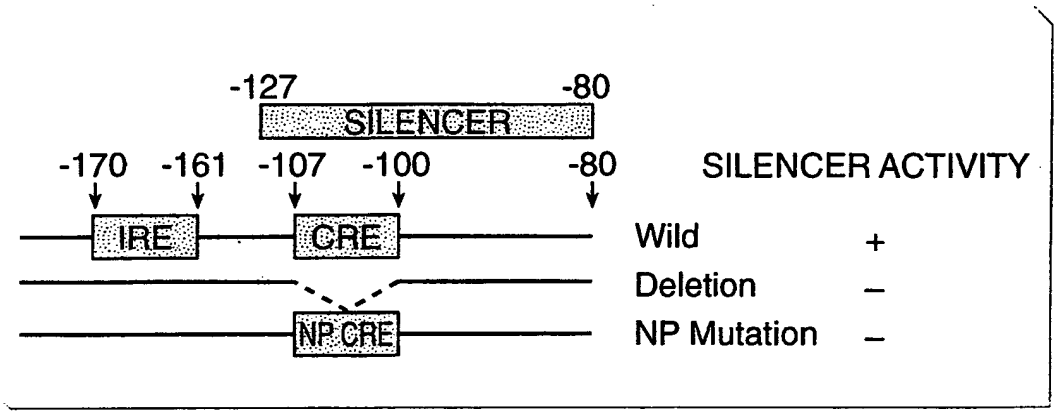


FIG. 41A



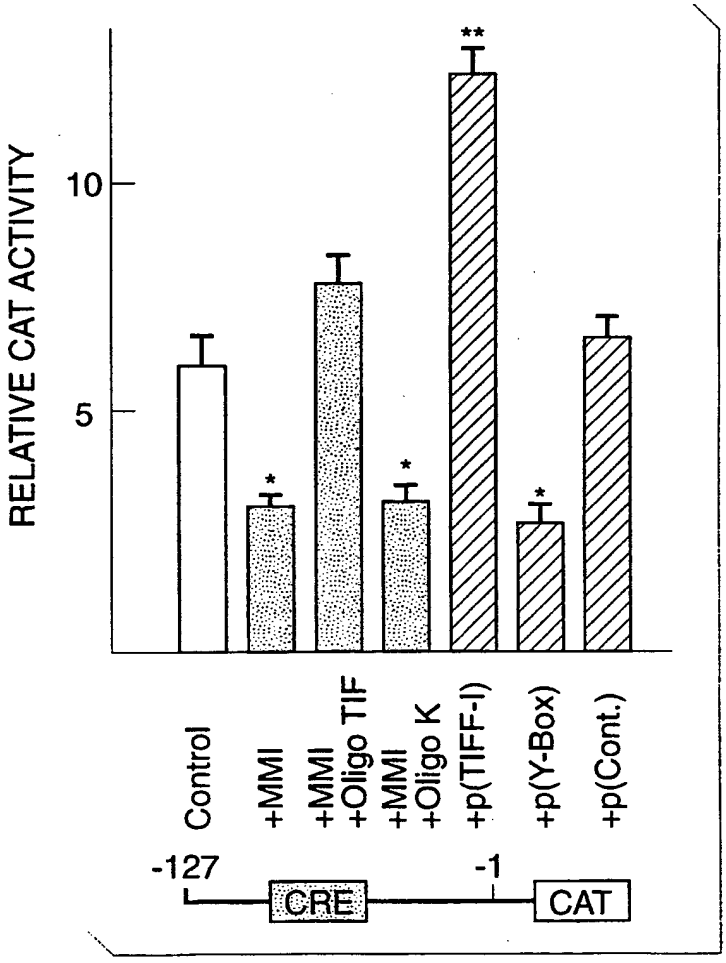


FIG. 41C

FIG. 41B

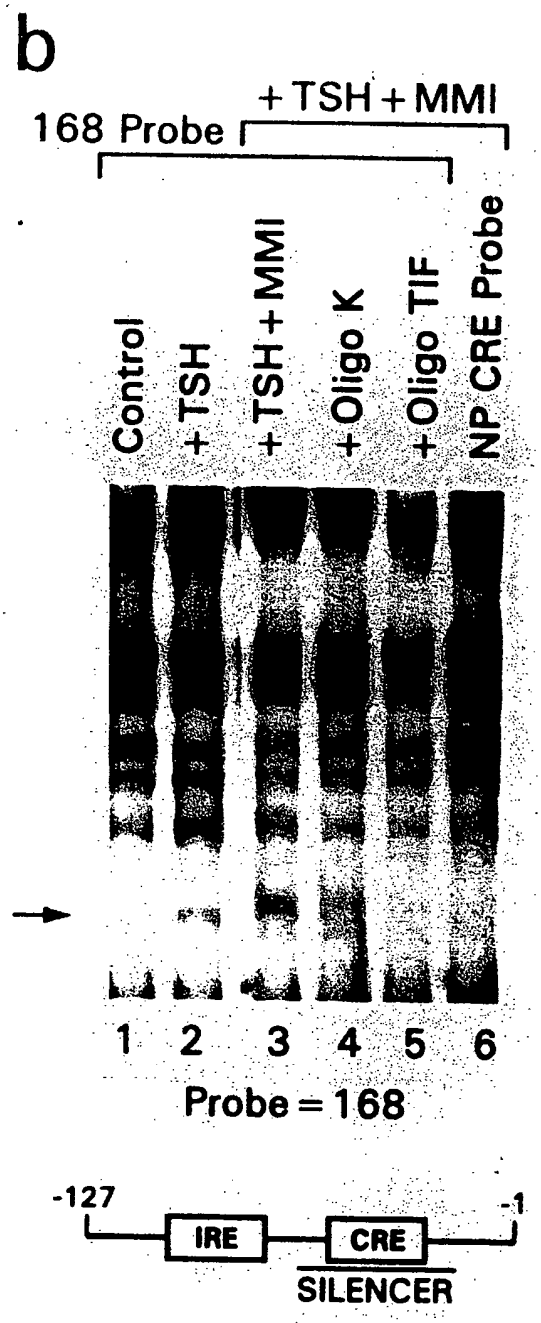
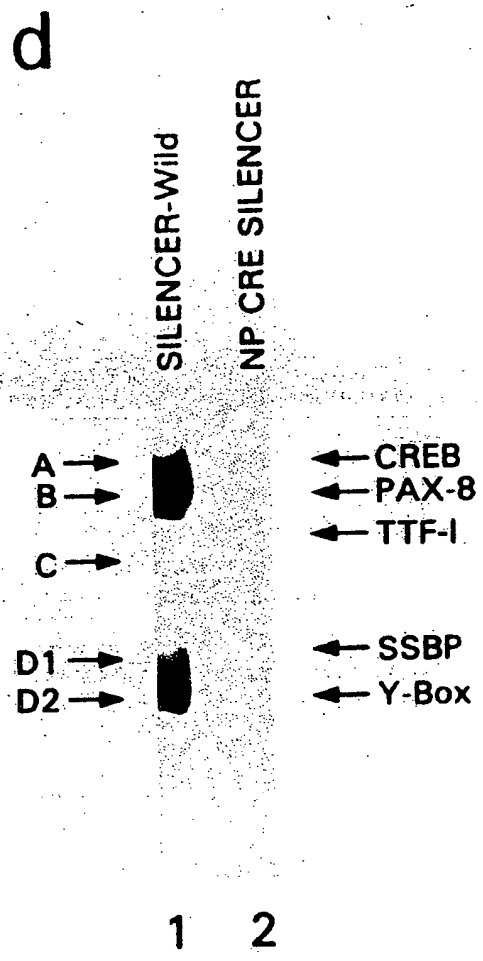

b
+TSH+MMI
168 Probe
Control
+TSH
+TSH+MMI
+Oligo K
+Oligo TIF
NP CRE Probe
1 2 3 4 5 6
Probe=168
-127
-1
IRE
CRE
SILENCER

FIG. 41D


d
SILENCER-Wild
NP CRE SILENCER
A
B
C
D1
D2
CREB
PAX-8
TTF-I
SSBP
Y-Box
1
2

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 96/13715

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 C12Q1/68 C07K14/47 C07K14/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO,A,94 28897 (SINGER DINAH S ET AL) 22 December 1994 cited in the application see the whole document	1,2, 4-13, 17-20,30 3,14-16, 18-21,31

X Y	EMBO J., vol. 12, no. 10, 1993, pages 3847-3854, XP002020724 WETERING VON ET AL.: "Sox-4, an sry-like HMG box protein, is a transcriptional activator in lymphocytes" see the whole document	22,24, 26-29 3,16,19, 31

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
16 December 1996	10.01.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hagenmaier, S

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 96/13715

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.BIOL.CHEM., vol. 265, no. 36, 25 December 1990, pages 22143-22152, XP002020723 OZER ET AL.: "Isolation and characterization of a cDNA clone for the CCAAT transcription factor EFIa reveals a novel structural motif" cited in the application	23,25-29
Y	see the whole document	3,16,19, 31

X	WO,A,92 04033 (GEN HOSPITAL CORP) 19 March 1992 cited in the application	30,31
A	See especially page 10	1-29

Y	PNAS, March 1992, pages 1944-1948, XP002021230 SAJI ET AL.: "Hormonal regulation of major histocompatibility complex class I genes in rat thyroid FRTL-5 cells : Thyroid-stimulating hormone induces a cAMP-mediated decrease in class I expression" cited in the application see the whole document	3,16,19

Y	MOL.ENDOCRINOL., vol. 9, May 1995, pages 527-539, XP000615281 SHIMURA ET AL.: "Single strand DNA-binding proteins and thyroid transcription factor-1 conjointly regulate thyrotropin receptor gene expression" see the whole document	3,16,19

Y	EMBO J., vol. 12, no. 8, 1993, pages 3163-3169, XP002020725 HOWCROFT ET AL.: "MHC class I gene expression is negatively regulated by the proto-oncogene, c-jun" see the whole document	3,19

Y	J.BIOL.CHEM., vol. 270, no. 19, 12 May 1995, pages 11453-11462, XP002020726 GIULIANI ET AL.: "Hormonal modulation of major histocompatibility complex class I gene expression involves an enhancer A-binding complex consisting of fra-2 and the p50 subunit of NF-kB" see the whole document	3,19

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ıal Application No
PCT/US 96/13715

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MOLECULAR CLONING, A LABORATORY MANUAL, 1989, pages 7.3-7.84, XP002020727 SAMBROOK ET AL.: "Extraction,purification, and analysis of messenger RNA from eukaryotic cells " see the whole document ---	14,15
Y	J.BIOL.CHEM., vol. 268, no. 15, 25 May 1993, pages 11380-11388, XP002020728 HAYASHI ET AL.: "Oxidoreductive regulation of nuclear factor kB" cited in the application see the whole document ---	18-21
A	IMMUNOLOGY TODAY, vol. 11, no. 8, 1990, pages 286-292, XP002020729 DAVID-WATINE ET AL.: "The regulation and expression of MHC class I genes" see the whole document -----	1-31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/ 13715

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 30,31
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 30,31 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/ composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

1. Claim 1-21: Methods for evaluating the ability of drugs to modulate MHC class I expression and for assessing the therapeutic potential thereof.

2. Claim 22,24 (complete) 26-29 (partial): Isolated nucleic acid sequence of Sox-4 protein, expression vectors comprising said nucleic acid and host organisms transformed with said vectors as well as recombinant and purified Sox-4 protein and antibodies reacting with said protein.

3. Claim 23,25 (complete) 26-29 (partial): Isolated nucleic acid sequence of Y-Box protein, expression vectors comprising said nucleic acid and host organisms transformed with said vectors as well as recombinant and purified Y-Box protein and antibodies reacting with said protein.

4. Claim 30,31: Preventing or treating transplantation rejection by using genetically modified cells expressing a nucleic acid sequence capable of MHC Class I suppression.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte nal Application No
PCT/US 96/13715

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9428897	22-12-94	AU-A-	7056494	03-01-95
		CA-A-	2164641	22-12-94
		EP-A-	0702554	27-03-96
		US-A-	5556754	17-09-96
WO-A-9204033	19-03-92	US-A-	5283058	01-02-94
		AU-B-	656150	27-01-95
		AU-A-	8510491	30-03-92
		CA-A-	2090009	01-03-92
		EP-A-	0550482	14-07-93
		JP-T-	6503810	28-04-94